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par **William RIBOULET**

LE RAT ZSF1: UN MODELE DE MALADIE CARDIO-RENALE ASSOCIEE AU SYNDROME METABOLIQUE. CARACTERISATION PAR L'UTILISATION D'UN ANTIOXYDANT, D'UN ANTAGONISTE DES RECEPTEURS DES MINERALOCORTICOÏDES ET D'UN INHIBITEUR DE L'ALDOSTERONE SYNTHASE.

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SUMMARY

"ZSF1 rat: a model of chronic cardiac and renal diseases in the context of metabolic syndrome. Characterization with anti-oxidant, mineralocorticoid receptor antagonist and aldosterone synthase inhibitor."

In the context of metabolic syndrome, development of Type 2 Diabetes is associated with (and influenced by) cardiac and renal comorbidities linked to micro- and macro-vasculature alterations. To assess the efficacy of new compounds on targeted organs in the context of metabolic syndrome, the Zucker fatty/Spontaneously hypertensive heart failure F1 hybrid (ZSF1) rat model could be suitable assuming cardiorenal alterations would develop in a short timeframe. Actually, the ZSF1 rat model recapitulates features of human metabolic syndrome, but develops relatively late (1year-time) and moderate cardiac and renal dysfunctions. The aim of our work was to exacerbate cardiorenal impairments in terms of onset and extent. Two options were explored. On one hand, unilateral nephrectomy was performed in ZSF1 rats, and cardiac and renal functions were longitudinally assessed. This surgical insult only significantly deteriorated renal function, which was prevented by standard of care, lisinopril and new renal protective agent, bardoxolone. On the other hand, subcutaneous infusion of angiotensin II (AngII) was used in the aim to induce hemodynamic and hormonal stress and thus to enhance cardiorenal impairments. AngII-infused ZSF1 rats displayed significant hypertension and increased levels of circulating aldosterone. Moreover, renal and cardiac functions dropped, concomitantly. We showed in this model that an aldosterone synthase inhibitor induced overall better renal protection than the mineralocorticoid receptor antagonist eplerenone. Our data showed that ZSF1-AngII rat is a suitable model to evaluate the cardio and renoprotective effects of drugs in the context of metabolic syndrome.

Keywords: ZSF1, aldosterone synthase inhibitor, metabolic syndrome, unilateral nephrectomy, angiotensin II.

RESUME

"Le rat ZSF1: un modèle de maladie cardio-rénale associée au syndrome métabolique. Caractérisation par l'utilisation d'un antioxydant, d'un antagoniste des récepteurs des minéralocorticoïdes et d'un inhibiteur de l'aldostérone synthase."

Chez les patients présentant un syndrome métabolique, le développement des comorbidités cardiaques et rénales associées au diabète de type 2 sont liées à des altérations au niveau de la micro- et macro-vasculature. Afin d'évaluer l'effet protecteur rénal et cardiaque de nouvelles molécules, le modèle de rat Zucker fatty/Spontaneously hypertensive heart failure F1 hybrid (ZSF1) semblait approprié. Cependant, son développement lent et les impacts rénaux et cardiaques modestes en limitaient son utilisation. Le but de nos travaux a consisté à exacerber et accélérer ces altérations par deux méthodes distinctes. Nous avons tout d'abord effectué une néphrectomie unilatérale chez le rat ZSF1 et évalué l'évolution des fonctions cardiaque et rénale en fonction de l'âge. Cette méthode n'a permis de mettre en évidence qu'une exacerbation de la dysfonction rénale, mais nous a néanmoins permis de démontrer l'effet protecteur rénal de l'inhibiteur de l'enzyme de conversion de l'angiotensine lisinopril ainsi que d'un composé antioxydant, le bardoxolone. Notre seconde stratégie a consisté à infuser de l'angiotensine II (AngII) durant 6 semaines, à des rats ZSF1. La conséquence de cette intervention a été un développement de l'hypertension déjà existante dans ce modèle, ainsi qu'une élévation significative du niveau d'aldostérone circulante. Dans ce contexte, les fonctions cardiaque et rénale ont été fortement dégradées. Enfin nous avons montré que dans ce modèle, un inhibiteur de l'aldostérone synthase induisait une meilleure protection rénale que l'antagoniste des récepteurs à l'aldostérone, éplérénone. Par l'ensemble des travaux effectués, nous avons mis en évidence que le rat ZSF1-AngII est un modèle de dysfonction cardio-rénale permettant d'évaluer l'effet protecteur de composés sur les fonctions rénale et cardiaque, dans un contexte de syndrome métabolique.

Mots-clés: ZSF1, inhibiteur de l'aldostérone synthase, syndrome métabolique, néphrectomie unilatérale, angiotensine II.

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LIST OF ABBREVIATIONS

11-DOC:	11-deoxycorticosterone
AACE:	American Association of Clinical Endocrinologists
ACE:	Angiotensin Converting Enzyme
ACE2:	Angiotensin Converting Enzyme 2
ADA:	American Diabetes Association
AGEs:	Advanced Glycation End products
AHA:	American Heart Association
Akt:	Protein Kinase B
ALLHAT:	Antihypertensive and Lipid-Lowering treatment to prevent Heart Attack Trial
ALPINE:	Antihypertensive Treatment and Lipid Profile in the North of Sweden Efficacy Evaluation
AngI:	Angiotensin I
AngII:	Angiotensin II
AngIII:	Angiotensin III
AngIV:	Angiotensin IV
AP-1:	Activator protein11
ApoB:	Apolipoprotein B
ARB:	Angiotensin Receptor Blocker
ASi:	Aldosterone Synthase Inhibitor
AT1:	Type 1 angiotensin II receptor
AT2:	Type 2 angiotensin II receptor
AT3:	Type 3 angiotensin II receptor
AT4:	Type 4 angiotensin II receptor
BEACON:	Bardoxolone methyl EvAluation in patients with Chronic kidney disease and type 2 diabetes mellitus: the Occurrence of renal eveNts
BMI:	Body Mass Index
CETP:	Cholesterol Ester Transport Protein
CKD:	Chronic Kidney Disease
COX-2:	Cyclooxygenase-2
CYP11B2:	Aldosterone Synthase / Cytochrome P450, family 11, subfamily B, polypeptide 2
Cyst-C:	Cystatin C
DASH:	Dietary Approaches to Stop Hypertension
DEMAND:	Developing Education on Microalbuminuria for Awareness of renal and cardiovascular risk in Diabetes
DRI:	Direct Renin Inhibitor; MRA, mineralocorticoid receptor antagonist
ECDCDM:	Expert Committee on the Diagnosis and Classification of Diabetes Mellitus
ECM:	Extracellular Matrix
EGIR:	European Group for the Study of Insulin Resistance
eNOS:	Endothelial NO Synthase
ERK:	Extracellular-signal Regulated Kinase
ET-1:	Endothelin-1
FFA:	Free Fatty Acids
FoxO1:	Forkhead box protein O1
GDF-15:	Growth Differentiation Factor-15
GFR:	Glomerular Filtration Rate
GLUT-4:	Glucose Transporter 4
HbA1C:	Glycated hemoglobin
HDL:	High Density Lipoprotein
HDL-C:	High Density Lipoprotein Cholesterol
ICAM-1:	Intracellular Cell Adhesion Molecule-1

IDF:	International Diabetes Federation
IFG:	Impaired Fasting Glucose
IGT:	Impaired Glucose Tolerance
IKK-β:	Inhibitor of nuclear factor Kappa-B Kinase subunit Beta
ILs:	Interleukins
IRS-1:	Insulin Receptor Substrate-1
JNC-VIII:	Eighth Joint National Commission
JNK:	c-Jun amino-terminal Kinase
KDIGO:	Kidney Disease: Improving Global Outcomes
KDOQI:	Kidney Disease Outcomes Quality Initiative
KIM-1:	Kidney injury molecule
LDL:	Light Density Lipoprotein
LV:	Left Ventricle
Mas-R:	Mas-receptor
MCP-1:	Monocyte Chemo-attractant Protein-1
MR:	Mineralocorticoïd Receptor
NADPH:	Nicotinamide Adenine Dinucleotide Phosphate
NCEP ATP III:	National Cholesterol Education Program – Third Adult Treatment Panel
NCEP:	National Cholesterol Education Program
NEFA:	Non Esterified Fatty Acids
NFkB:	Nuclear Factor-kappa B
NGT:	Normal Glucose Tolerance
NHANES:	National Health and Nutrition Examination Survey
NIH:	National Institute of Health
NO:	Nitric Oxide
$O2^{-}$:	Superoxide anion
$ONOO^{-}$:	Peroxynitrite
OSA:	Obstructive Sleep Apnea
PAI-1:	Plasminogen Activator Inhibitor-1
PARP:	Poly ADP Ribose Polymerase
PGI2:	Prostacyclin
PGIS:	Prostacyclin Synthase
PI3-kinase:	Phosphoinositide 3 kinase
PKC:	Protein Kinase C
PPAR:	Peroxisome Proliferator Activated Receptor
(P)RR:	Prorenin Receptor
RAAS:	Renin Angiotensin Aldosterone System
RAGE:	Receptors for Advanced Glycation End product
RNA:	Ribonucleic Acid
ROS:	Reactive Oxygen Species
SGK1:	Serum and Glucocorticoid-regulated Kinase 1
SHHF:	Spontaneously Hypertensive Heart Failure
SRAA:	Système Rénine Angiotensine Aldostérone
STOP-NIDDM:	STOP-Noninsulin-Dependent Diabetes Mellitus
SUN:	Seguimiento University of Navarra
T2D:	Type 2 Diabetes
T2DM:	Type 2 Diabetes Mellitus
TA2:	Thromboxane A2
TF:	Tissue Factor
TGF-β:	Transforming Growth Factor-β
TGs:	Triglycerides
TLR:	Toll Like Receptor

TNFα:	Tumor Necrosis Factor α
TZD:	Thiazolidinedione
UKPDS:	UK Prospective Diabetes Study
UNx:	Unilateral Nephrectomy
USA:	United States of America
V-ATPase:	Vacuolar H+-Adenosin Tri-Phosphatase
VCAM-1:	Vascular Cell Adhesion Molecule-1
VEGF:	Vascular Endothelial Growth Factor
VLDL:	Very Light Density Lipoprotein
WC:	Waist Circumference
WHO:	World Health Organization
ZDF:	Zucker Diabetic Fatty
ZSF1:	Zücker fatty/Spontaneously hypertensive heart failure F1 hybrid
β2M:	β2 Microglobulin

INTRODUCTION GENERALE

Le syndrome métabolique est caractérisé par une inflammation chronique accompagnée d'un risque accru de développer un diabète de type II et des maladies cardiovasculaires. Ce syndrome se définit par la combinaison de différents symptomes tels que la résistance à l'insuline, la dyslipidémie, une dysfonction endothéliale, l'hypertension ou encore le diabète de type 2 (Kaur 2014). Le diabète est une condition physiopathologique associée à d'importantes complications au niveau de la microvasculature et des gros vaisseaux. Les complications microvasculaires incluent le rétinopathie, la néphropathie, et la neuropathie; les complications au niveau des gros vaisseaux concernent les vaisseaux périphériques, et sont représentées par les maladies coronariennes ou encore les accidents vasculaires cérébraux, induisant des dommages tissulaires chez environ un tiers à la moitié des patients diabétiques (Cade 2008). Avec une prévalence du syndrome métabolique qui ne cesse d'augmenter, il est important et nécessaire d'établir de nouvelles approches thérapeutiques permettant aux patients concernés de voir leur statut métabolique amélioré. Etablir des modèles animaux (et notamment chez le rongeur) de syndrome métabolique n'est pas chose aisée compte-tenu de la complexité des maladies associées. Différents modèles ont été caractérisés, mais ne développent pas l'ensemble des symptomes définissant le syndrome métabolique, ce qui constitue un important fossé avec la réalité du syndrome retrouvé chez l'Homme. Au début des années 2000, le rat ZSF1 (Zücker fatty/Spontaneously hypertensive heart failure F1 hybrid) s'est montré prometteur dans ce domaine, compte-tenu du fait qu'il partage des caractéristiques communes avec le développement du syndrome humain. Ce modèle de rat obèse devient donc résistant à l'insuline, présente une hypertension modérée, et développe une néphropathie diabétique ainsi qu'une cardiomyopathie (Tofovic and Jackson 2003; Griffin *et al.* 2007; Joshi *et al.* 2009). Néanmoins, le degré de sévérité de ces maladies cardiaque et rénale associées est assez limité, et leur apparition ne survient que tardivement. Par consequent, ce modèle n'est pas forcément adapté à l'étude des effets cardio- et rénoprotecteur de nouveaux composés dans un contexte de syndrome métabolique en un temps optimal. Le but de ce travail a été d'améliorer les défauts majeurs de ce modèle de rat ZSF1 en augmentant le degré de sévérité de la maladie cardiorénale associée au syndrome métabolique, ainsi

que d'en réduire le temps de développement en vue de son utilisation pour la caractérisation des effets protecteurs de nouvelles molécules au niveau cardiaque et rénal.

En premier lieu, nous reviendrons sur les différentes composantes définissant le syndrome métabolique et leurs impacts notamment au niveau des fonctions rénale et cardiaque. Ensuite nous décrirons la manière dont nous avons évalué la fonction cardiaque du modèle ZSF1 au cours du temps, et évoquerons les connaissances actuelles sur l'évolution de leur fonction rénale. Dans une seconde partie nous développerons les deux stratégies utilisées pour exacerber les défaillances rénale et cardiaque chez ces animaux et ainsi améliorer le modèle: la première par néphrectomie unilatérale (approche chirurgicale), la seconde par l'infusion d'angiotensine II (approche hormonale). Enfin nous montrerons que ces approches ont permis d'améliorer le modèle ZSF1, et donc de tester la capacité à protéger la fonction rénale et/ou cardiaque, selon la méthode utilisée, de différents composés: le bardoxolone (antioxydant), l'eplerenone (antagoniste des récepteurs aux minéralocorticoïdes), et enfin un inhibiteur sélectif de l'aldostérone synthase.

GENERAL INTRODUCTION

Metabolic syndrome is a state of chronic low-grade inflammation that increases the risk of cardiovascular diseases and development of type 2 diabetes. This is recognized as a combination of pathophysiological disorders such as insulin resistance, dyslipidemia, endothelial dysfunction, genetic susceptibility, hypertension, hypercoagulable state, and chronic stress, and type 2 diabetes (Kaur 2014). Diabetes has been reported to be strongly associated with microvascular and macrovascular complications. Microvascular complications include retinopathy, nephropathy, and neuropathy, whereas macrovascular ones include ischemic heart disease, peripheral vascular disease, and cerebrovascular disease, resulting in organ and tissue damage in approximately one third to one half of people with diabetes (Cade 2008). With an increasing global prevalence of metabolic syndrome, it's necessary to work on new therapeutics that could improve the metabolic status of patients. As a cluster of metabolic disturbances, animal models (especially rodents) that mimic the onset of metabolic syndrome do not exist *per se* and are not easy to set up; several models have been characterized, with only focus on one of the component of metabolic syndrome listed above. This constitutes a gap with the human situation. Quite recently, the ZSF1 (Zücker fatty/Spontaneously hypertensive heart failure F1 hybrid) rat has given some hope in the field of metabolic syndrome, as it resumes features and complications that are common with the human situation. It is characterized by insulin resistance, mild hypertension, obesity and develops diabetic nephropathy and cardiomyopathy (Tofovic and Jackson 2003; Griffin *et al.* 2007; Joshi *et al.* 2009). Nevertheless, the associated cardio-renal defects observed with metabolic syndrome are quite limited and develop on the long run only. In this condition it is not a suitable model to efficiently characterize new compounds, especially preventing cardiac and renal failure associated with metabolic syndrome in a reasonable time frame. The goal of the present work is to worsen and shorten the time frame of the cardio-renal defects in the ZSF1 model in order to facilitate new drugs' expertise.

In the first chapter of this thesis work, we will describe the components of the metabolic syndrome and their consequences on health status, especially the way they impact cardiac and renal functions. Then, we

will describe how we longitudinally characterized cardiac function of the ZSF1 rat. In a second chapter we will develop how we improved (worsened) the ZSF1 rat model by two different approaches, *i.e.* surgical insult (by unilateral nephrectomy) and hormonal stress (by infusion of angiotensin II). These approaches worsened extent and onset of heart and kidney defects. Protective actions of compounds such as bardoxolone (anti-oxidant), eplerenone (mineralocorticoid receptor antagonist), and finally an in-house aldosterone synthase inhibitor, were explored. The treatments demonstrated efficacy in protecting the structure and function of kidney, and heart in a lesser extent.

1. BIBLIOGRAPHY

Ce premier chapitre bibliographique décrit en premier lieu la difficulté qu'a eue la communauté scientifique à trouver un consensus quant à la définition du syndrome métabolique, étant donné que ce n'est pas une maladie en soi, mais plutôt une combinaison de plusieurs altérations touchant différents organes. Un consensus ayant été adopté et proposé par l'International Diabetes Federation, nous décrirons les différentes composantes associées à ce syndrome, à savoir l'obésité, le diabète de type 2 et ses différentes étapes de développement, et comment elles mènent à la notion de résistance à l'insuline, centrale dans ce syndrome. L'hyperglycémie étant une conséquence du diabète de type 2 et de la résistance à l'insuline, nous décrirons dans quelle mesure elle est génératrice de complications au niveau des vaisseaux sanguins, et notamment au niveau endothélial par la production d'espèces réactives de l'oxygène. Nous poursuivrons par les conséquences pathologiques associés: une inflammation vasculaire, l'athérosclérose, et une vasoconstriction. L'hypertension induite est aussi une composante importante du syndrome métabolique, et est associée à l'obésité, au diabète, à la résistance à l'insuline, et à l'activation pathologique du système rénine angiotensine aldostérone (SRAA). Toutes ces altérations ont une incidence néfaste sur les fonctions rénale et cardiaque, et les patients présentant un syndrome métabolique peuvent être sujets au développement d'une néphropathie diabétique pouvant mener à une maladie rénale chronique voire à une insuffisance rénale, ainsi qu'à des risques cardiovasculaires accrus.

L'implication du SRAA est importante dans le syndrome métabolique, c'est pourquoi il est également décrit dans cette partie bibliographique, au niveau systémique, mais également tissulaire. Il est présenté dans le contexte du développement du syndrome, par exemple par son action sur la résistance à l'insuline, et sur l'hypertension. De plus, les effets de deux hormones clés du SRAA, l'angiotensine II (AngII) et l'aldostérone sont détaillés, ainsi que les stratégies pharmacologiques adoptées pour contrer les effets néfastes du SRAA sur le syndrome métabolique. De manière plus large enfin, nous développerons la manière dont ce syndrome est pris en charge chez les patients pour tenter de le réduire.

Pour faire le lien avec la recherche pharmaceutique, les différents modèles animaux disponibles pour étudier le syndrome métabolique ou tout du moins certaines composantes sont évoqués, ce qui a donné lieu à la publication d'un éditorial. Il en ressort que le modèle de rat ZSF1 nous a semblé le plus pertinent et a constitué l'objet de nos études, développées dans le second chapitre expérimental de ce manuscrit.

1.1 METABOLIC SYNDROME HISTORY AND DEFINITION

Metabolic syndrome is not a disease by itself but is defined by a cluster of metabolic disturbances which represent major risk factors for cardiovascular disease. These several abnormalities have already been observed and studied during the First World War, by two physicians, Karl Hitzenberger and Martin Richter-Quittner. They made clinical observations of metabolic abnormalities in patients and discussed the relationship between metabolism and vascular hypertension (Hitzenberger and Richter-Quittner 1921), as well as blood pressure and type 2 diabetes (Hitzenberger 1921). Then rapidly, two other physicians, Kylin and Marañon described in two different papers the coexistence of hypertension and type two diabetes in adults and defined mechanisms for the development of these disorders (Kylin 1921; Marañon 1922). In 1923, Kylin described for the first time the existence of a hypertension–hyperglycemia–hyperuricaemia syndrome (Kylin 1923). Then the notion of insulin resistance in patients with type 2 diabetes was pointed out by Himsworth in 1936 (Himsworth 1936). This notion will be later central in the understanding of the metabolic syndrome pathophysiology. More than 10 years later, the difference between android and gynaecoïd obesity was described and the link between the android form and diabetes, hypertension, gout and atherosclerosis were evoked (Vague 1947; Vague 1956). Then during the 1960s, two important points were described: the disturbances were associated with lifestyle and nutrition habits (Mehnert and Kuhlmann 1968), and the role of free fatty acids in the development of insulin resistance and type 2 diabetes were evoked (Randle *et al.* 1963). Despite the important research in this field, the name “metabolic syndrome” only appeared in 1977 with Haller (Haller 1977), and was used again in 1981 to talk about the association of type 2 diabetes, hyperinsulinemia, obesity, hypertension, hyperlipidemia, gout and thrombophilia (Hanefeld and Leonhardt 1981). In 1988, Reaven proposed the name “syndrome X” (Reaven 1988) to emphasize the fact that it was quite misunderstood, in which insulin resistance was central in the etiology. Importantly, one year later, Kaplan talked about the “deadly quartet” that included central obesity for the first time, on top of hypertriglyceridemia, hypertension, glucose intolerance (Kaplan 1989). Then many scientists contributed to several definitions of the metabolic syndrome, and some

talked about “insulin resistance syndrome” (DeFronzo and Ferrannini 1991; Haffner *et al.* 1992) with more or less the same criteria. This shows that at this time, still no international consensus had been found about what was included in the syndrome, despite it was already an important research topic in the 1990s (Sarafidis and Nilsson 2006). Nowadays, “metabolic syndrome” is the most common term used. Still, defining metabolic syndrome remains difficult, as several institutions have successively proposed definitions (Table 1). The World Health Organization (WHO) has suggested in 1999 a first working definition that was to be improved with time, and aimed to define the essential components of the syndrome and importantly the insulin resistance. Unfortunately this definition was very complex and inconvenient to work with. Less than one year later, the European Group for the Study of Insulin Resistance (EGIR) modified the WHO definition, insisted more on the abdominal obesity and skipped the type 2 diabetes component (Balkau and Charles 1999). Only two years later, the National Cholesterol Education Program – Third Adult Treatment Panel (NCEP ATP III) simplified the metabolic syndrome definition, with notably several differences with the WHO definition, such as insulin resistance which was not considered as mandatory anymore, or central obesity instead of abdominal obesity (NCEP, 2002). In a clinical point of view, this definition was much more practicable as the one from WHO: for example, albuminuria was no longer required to be measured. In 2003, the American Association of Clinical Endocrinologists (AACE) proposed to place impaired glucose tolerance, dyslipidemia, obesity and hypertension as the main components of metabolic syndrome (moreover they preferred the term “insulin resistance syndrome” as EGIR did), with clinical judgment as an important part. Most of worldwide researchers still preferred using the NCEP ATP III definition as it was the simplest and the more clinically relevant (Parikh and Mohan 2012). However, waist circumference cutoffs used in this definition were not adapted to many countries because of the morphology of the populations (Tan *et al.* 2004; Matsuzawa 2005; Zhou *et al.* 2005; Barbosa *et al.* 2006).

With all these controversies regarding diagnosis criteria and in the hope of finding a global definition, the International Diabetes Federation (IDF) proposed a worldwide consensus in 2005, as displayed in

Table 1 (IDF, 2005). It is stated in the definition that although the pathogenesis of each component is complex and not fully understood, **central obesity** and **insulin resistance** are key causative factors of metabolic syndrome. Nevertheless, although **insulin resistance is central in this syndrome is not an essential requirement** as it is not easy to assess in a daily clinical practice.

Table 1 **Evolution of the diagnostic criteria proposed for the clinical diagnosis of the metabolic syndrome.**

From (Kaur 2014).

Clinical measures	WHO (1998)	EGIR (1999)	ATPIII (2001)	AACE (2003)	IDF (2005)
Insulin resistance	IGT, IFG, T2DM, or lowered insulin Sensitivity[a] plus any 2 of the following	Plasma insulin >75th percentile plus any 2 of the following	None, but any 3 of the following 5 features	IGT or IFG plus any of the following based on the clinical judgment	None
Body weight	Men: waist-to-hip ratio >0.90; women: waist-to-hip ratio >0.85 and/or BMI > 30 kg/m^2	WC ≥94 cm in men or ≥80 cm in women	WC ≥102 cm in men or ≥88 cm in women	BMI ≥ 25 kg/m^2	Increased WC (population specific) plus any 2 of the following
Lipids	TGs ≥150 mg/dL and/or HDL-C <35 mg/dL in men or <39 mg/dL in women	TGs ≥150 mg/dL and/or HDL-C <39 mg/dL in men or women	TGs ≥150 mg/dL HDL-C <40 mg/dL in men or <50 mg/dL in women	TGs ≥150 mg/dL and HDL-C <40 mg/dL in men or <50 mg/dL in women	TGs ≥150 mg/dL or on TGs Rx. HDL-C <40 mg/dL in men or <50 mg/dL in women or on HDL-C Rx
Blood pressure	≥140/90 mm Hg	≥140/90 mm Hg or on hypertension Rx	≥130/85 mm Hg	≥130/85 mm Hg	≥130 mm Hg systolic or ≥85 mm Hg diastolic or on hypertension Rx
Glucose	IGT, IFG, or T2DM	IGT or IFG (but not diabetes)	>110 mg/dL (includes diabetes)	IGT or IFG (but not diabetes)	≥100 mg/dL (includes diabetes)[b]
Other	Microalbuminuria: Urinary excretion rate of >20 mg/min or albumin: creatinine ratio of >30 mg/g.			Other features of insulin resistance[c]	

[a]Insulin sensitivity measured under hyperinsulinemic euglycemic conditions, glucose uptake below lowest quartile for background population under investigation.
[b]In 2003, the American Diabetes Association (ADA) changed the criteria for IFG tolerance from >110 mg/dl to >100 mg/dl (Shaw *et al.* 2006).
[c]Includes family history of type 2 diabetes mellitus, polycystic ovary syndrome, sedentary lifestyle, advancing age, and ethnic groups susceptible to type 2 diabetes mellitus.
BMI: body mass index; HDL-C: high density lipoprotein cholesterol; IFG: impaired fasting glucose; IGT: impaired glucose tolerance; Rx: receiving treatment; TGs: triglycerides; T2DM: type 2 diabetes mellitus; WC: waist circumference.

As a result and according to this definition, a patient will be diagnosed with metabolic syndrome if he displays **central obesity plus two of the following components: raised triglycerides, reduced high density lipoproteins (HDL), raised fasting plasma glucose and hypertension** (Figure 1).

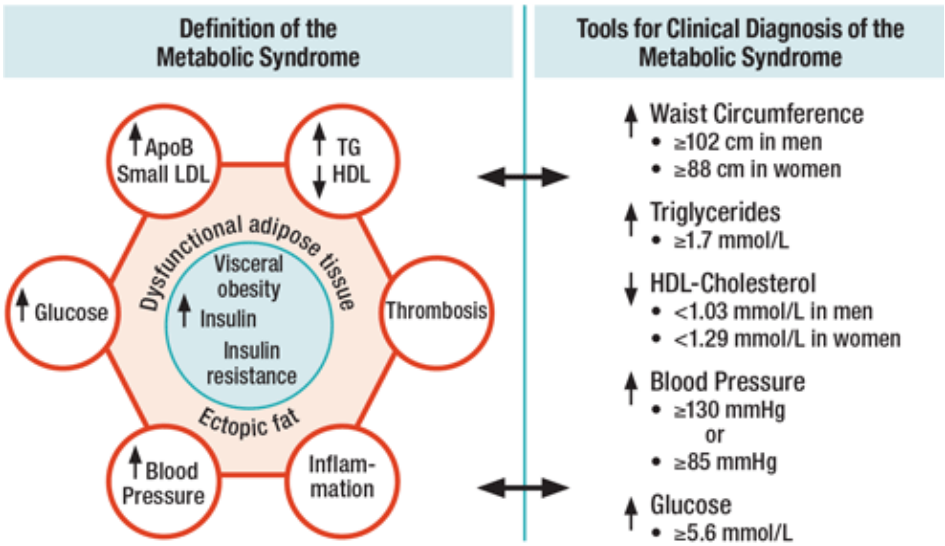

Figure 1 Schematic view of the Metabolic Syndrome.

From (Barker 2012) – ApoB: apolipoprotein B; HDL: high density lipoprotein; LDL: low density lipoprotein; TG: triglycerides.

Nevertheless, as the different ethnicities and populations have different norms for body weight, waist circumference, and also different risk to develop type 2 diabetes or cardiovascular disease, the IDF, has proposed a new set of criteria with ethnic/racial specific cut-offs in 2009 (Alberti *et al.* 2009) (Table 2).

Table 2 Gender and age-specific waist circumference cut-offs.

From (Alberti *et al.* 2009).

Country/ethnic group	Waist circumference cut-off	
	Male (cm)	Female (cm)
Europids In USA, the ATPIII values (102 cm males; 88 cm females) are likely to continue to be used for clinical purposes.	≥94	≥80
South Asians Based on a Chinese, Malay, and Asian-Indian population.	≥90	≥80
Chinese	≥90	≥80
Japanese	≥90	≥80
Ethnic South and Central Americans	Use South Asians recommendations until more specific data are available.	
Sub-Saharan Africans	Use European data until more specific data are available.	
Eastern Mediterranean and Middle East (Arabs) population	Use European data until more specific data are available.	

According to the definition used, and ethnicity, age, sex or region, the prevalence of metabolic syndrome can be estimated from about 10% to more than 80% of the population (Desroches and Lamarche 2007; Kolovou *et al.* 2007). Worldwide, the IDF estimates that 25% of the adult population has the metabolic syndrome (IDF 2005). The prevalence has been shown to be influenced by several factors such as sedentary lifestyle, genetic background, physical activity, smoking, education, heredity of diabetes (Cameron *et al.* 2004) and ageing (Ford *et al.* 2002; Park *et al.* 2003). The National Health and Nutrition Examination Survey (NHANES) has shown that 5% of the subjects of normal weight, 22% among the overweight, and 60% among the obese had metabolic syndrome (Park *et al.* 2003) showing in the meantime that some non-obese people can have the syndrome. Palaniappan et al. have shown that each 11 cm increase in waist circumference was associated with a risk of developing metabolic syndrome within 5 years by 80% (Palaniappan *et al.* 2004). Moreover, the risk of developing metabolic syndrome increases with the number of metabolic disorders (Andreadis *et al.* 2007).

In 2009, it was reported that metabolic syndrome induces a 5-fold increase in the risk of developing type 2 diabetes and a 2-fold increase in the risk for cardiovascular disease over the next 5 to 10 years (Alberti *et al.* 2009). More precisely, stroke risks are increased by 2 to 4 fold, myocardial infarction by 3 to 4 fold, and death event by 2 fold compared to age-matched healthy population (Alberti *et al.* 2005).

As indicated before, metabolic syndrome is not a disease by itself but is rather defined by a cluster of disturbances that we will develop in the next section.

1.2 OBESITY, INSULIN RESISTANCE AND TYPE 2 DIABETES

As stated previously, the worldwide accepted consensus on the definition of metabolic syndrome proposed by the IDF stated that developing insulin resistance is not an essential requirement as it is not easy to assess in a daily clinical practice. Nevertheless, it is also stated that **insulin resistance has been central in the development of this syndrome** (IDF 2014), which was also recognized by the former definitions we described in the previous chapter (Table 1).

In this section, we will define how insulin resistance develops in a context of obesity and dyslipidemia, both components of the metabolic syndrome, and the way it is linked to type 2 diabetes which leads to morbid consequences such as chronic kidney disease and cardiovascular disease.

1.2.1 Obesity

According to the World Health Organization, overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health. The main cause of obesity and overweight is an energy imbalance between calories consumed and calories expended, essentially due to an increased consumption of high fat diet with an increase in physical inactivity and sedentary lifestyle. These two conditions are defined by the Body Mass Index (BMI), calculated as a person's weight in kilograms divided by the square of his height in meters. People are considered as overweight if their BMI is greater than or equal to 25 and obese if greater than or equal to 30. The main advantage of this index is that it is independent of the sex and age, but it may not correspond to the same degree of fatness in different individuals. Raised BMI is a major risk factor to develop type 2 diabetes, among other diseases and disorders (WHO 2014).

Worldwide, between 1980 and 2013, prevalence of combined overweight and obesity rose by 27.5% for adults and 47.1% for children. The number of overweight and obese individuals was estimated at

857 million in 1980, and to be 2.1 billion in 2013. Worldwide, the proportion of overweight people increased from 28.8% in 1980, to 36.9% in 2013 for men, and from 29.8% to 38.0% for women. In 2013, the prevalence of obesity was higher in women in developed and developing countries than in men (Ng *et al.* 2014).

The increase in the prevalence of type 2 diabetes is closely linked to central obesity: about 90% of type 2 diabetes is attributable to excess weight. Furthermore, it has been shown that approximately 197 million people worldwide had impaired glucose tolerance in 2007, most commonly because of obesity and the associated metabolic syndrome (Hossain *et al.* 2007).

1.2.2 Insulin resistance and Type 2 diabetes

According to the IDF, there are 387 million people with diabetes worldwide today with 179 million of undiagnosed people; by 2035 this number is expected to rise to more than 592 million people. In 2014 diabetes caused 4.9 million deaths, *i.e.* 1 every 7 seconds (IDF 2014). Diabetes Mellitus or Type 2 Diabetes represent 90% of Diabetes and is recognized as a worldwide epidemic whose progression unhalted. Prevalence of obesity, genetic susceptibility, urbanization and ageing contribute to important increase of the number of people suffering from diabetes mellitus. The proportion of obese and diabetic patients might slow down or even decrease in Europe and the United States while it should increase in Asia and Africa (ECDCDM Balarajan and Villamor 2009; Doak *et al.* 2012; Flegal *et al.* 2012; Tabaei *et al.* 2012). We could hypothesize that way of life is changing in all these regions, but not in the same way: in Asia and Africa, physical inactivity and rich diet consumption habits may increase with the economic development, whereas population in the United States starts to increase physical activity again.

People suffering from type 2 diabetes can be asymptomatic for many years. Early symptoms of type 2 diabetes may include infections that are more frequent and slower healing process, fatigue, hunger, increased thirst and urination, blurred vision or erectile dysfunction. However, type 2 diabetes

diagnosis is often made at an advanced stage through blood or urine bio-analysis, *i.e.* when micro- and macro-vascular defects already exist (ECDCDM report 2003; Wild *et al.* 2004; Danaei *et al.* 2011). Among the diabetic population two main co-morbidities have be identified: cardiovascular diseases (coronary heart disease, stroke, peripheral artery disease due to atherosclerosis) and kidney failure associated with elevated proteinuria (Janghorbani *et al.* 2007; Arboix *et al.* 2008; Preiss *et al.* 2011; Barkoudah *et al.* 2012).

Diagnosis is then confirmed by several measurements such as:

- Fasting blood glucose level - diabetes is diagnosed if fasting blood glucose is higher than 126 mg/dL (7 mM) (when measured twice),

- Glycated hemoglobin (HbA1C) of 6.5% or higher, this notion of glycation will be further developed in the next section,

- During oral glucose tolerance test performed by drinking a special sugar drink, glycemia is higher than 200 mg/dL 2 hours after the glucose challenge (Pories *et al.* 2011) (American Diabetes Association 2013).

Several risk factors for development of type 2 diabetes exist, such as inactivity, central obesity, family history, age over 45 years, gestational diabetes, elevated glycemia, metabolic syndrome.

The onset of type 2 diabetes occurs mainly in people that are insulin resistant, have genetic predisposition, or in response to overweight generally as a consequence of sedentary lifestyle and over nutrition. Insulin resistance is defined as a pathophysiological condition in which a normal insulin concentration does not adequately produce a normal insulin response in the peripheral target tissues such as adipose tissue, muscle and liver, *i.e.* glucose uptake and storage is lowered in these organs, leading to high glycemia values.

With time, insulin resistance develops due to lipid accumulation (lipotoxicity) that can activate several pathways like unfolded protein response or immune pathways, which are linked to free fatty acids uptake, lipogenesis and energy expenditure. As a result, accumulation of lipid metabolites such as ceramides and diacyglycerols in skeletal muscles and liver impairs insulin signaling in the cells (Samuel and Shulman 2012).

Consequently, pancreatic β-cells in pancreatic Langerhans islets produce more insulin to compensate the impaired insulin signaling and to maintain euglycemia. This peculiar stage is called pre-diabetes, and is characterized by subjects who are still normally glucose tolerant. At this stage, exercise and appropriate diet can still reverse the process of β-cell signaling impairment. With time, if inactivity and overweight develops further, β-cells function tends to decline more; under these circumstances people evolve progressively towards impaired glucose tolerance, characterized by significant hyperglycemia, as insulin secretion signaling is impaired and targeted organs (mainly skeletal muscles) are desensitized (Kaur 2014). This vicious circle contributes progressively to β-cell failure. This is the step during which T2D is obviously diagnosed (Figure 2) (Kasuga 2006; Prentki and Nolan 2006).

In Langerhans islets, α-cells also have an important role, as they produce glucagon, a hormone that favors release of glucose in the blood. In type 2 diabetes, there's a dysregulation of glucagon release, which is not suppressed after a meal. Whether this is a primary cause or a consequence of β-cell dysfunction is still not clear (Dunning and Gerich 2007).

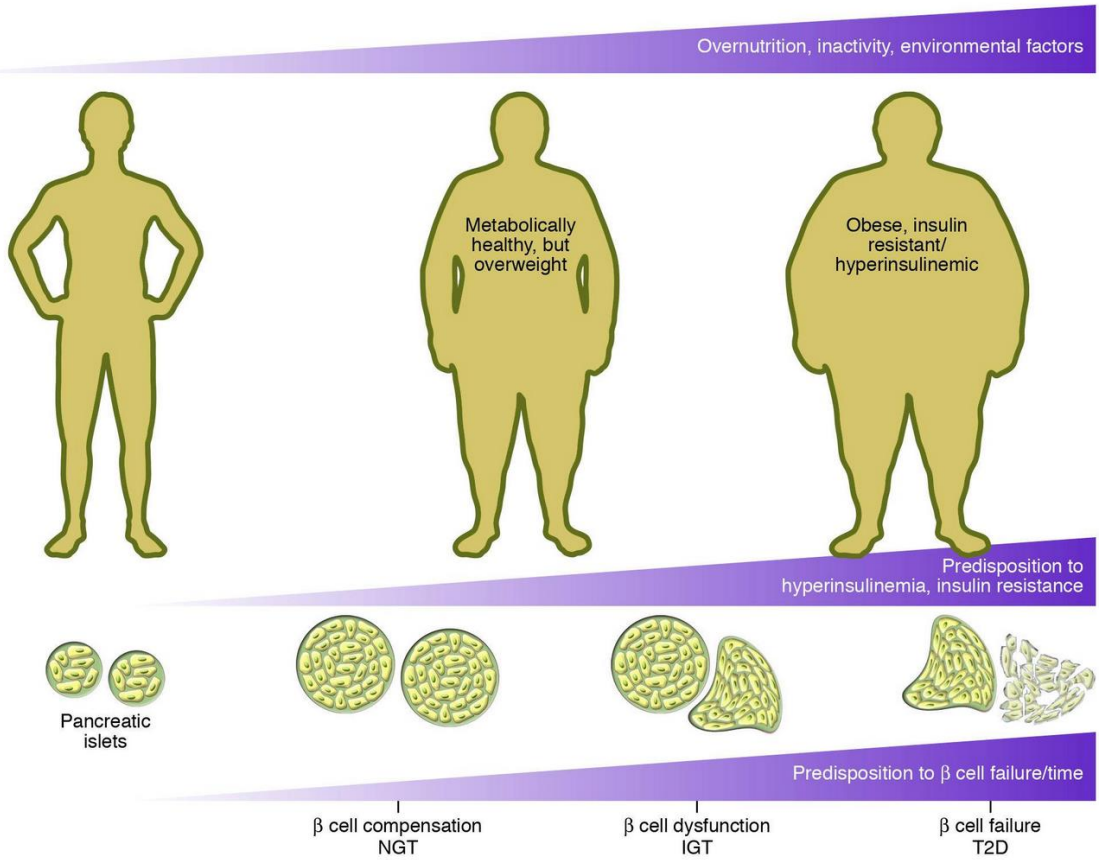

Figure 2 Islet β-cell failure and the natural history of type 2 diabetes.

From (Prentki and Nolan 2006) – IGT: impaired glucose tolerance; NGT: normal glucose tolerance; T2D: type 2 diabetes.

As stated before, lipotoxicity plays an important role in the development of insulin resistance. In adipocytes, lipolysis is increased, which induces elevation of free fatty acids levels.

On one hand, the increased levels of free fatty acids also activate toll like receptor (TLR). These receptors have complex expression patterns in different cell types but their expression is particularly significant in different types of white blood cells like mast cells, macrophages, and dendritic cells (Christmas 2010). TLR are known to recognize pathogen-associated molecular patterns like the lipopolysaccharide of Gram-negative bacteria and activate the innate immune system and provoke inflammation. In particular, TLR4 signaling has been shown to be pivotal in the pathological lipid effects on cardiovascular and metabolic functions (Eguchi and Manabe 2014). As a result, TLR can be activated by free fatty acids, that:

- induce, *via* nuclear translocation of nuclear factor-kappa B (NF-kB), up-regulation of the genes coding for interleukin 6 (IL-6) and tumor necrosis factor α (TNFα), and as a result induce a pro-inflammation status. TNFα has been reported to increase IL-18 that has a direct link with the development of insulin resistance (Krogh-Madsen *et al.* 2006).
- induce phosphorylation of insulin receptor substrate-1 (IRS-1) *via* c-Jun amino-terminal kinase (JNK) and protein kinase C (PKC), blocking its action on phosphoinositide 3 kinase (PI3-kinase) and Akt that normally enhances the glucose transporter 4 (GLUT-4) synthesis necessary to internalize glucose into the cell (essentially expressed on skeletal muscle and adipose tissue). This results in insulin resistance (Figure 3) (Paneni *et al.* 2013).

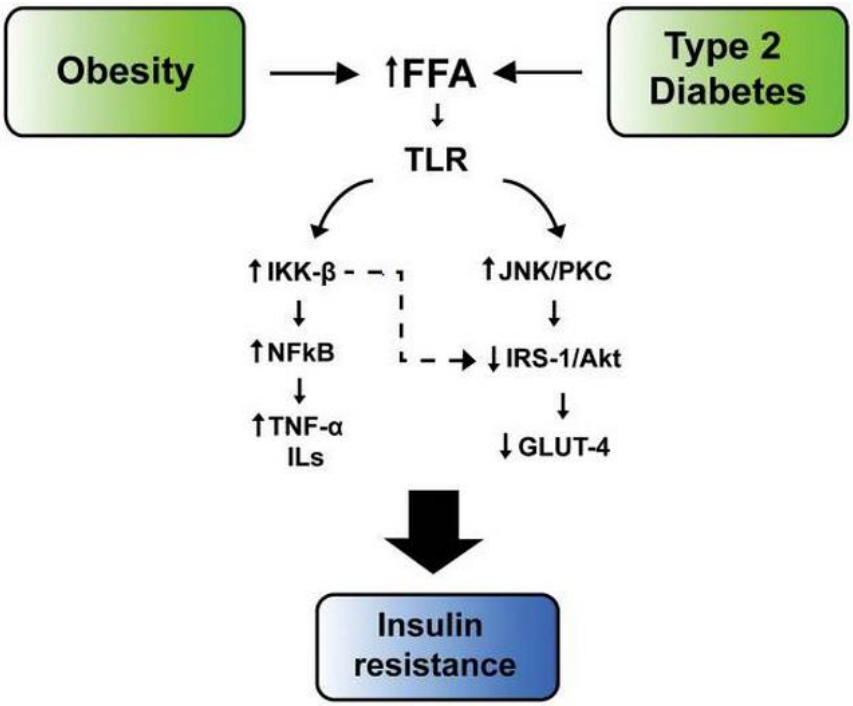

Figure 3 Mechanisms leading to insulin resistance.

Adapted from (Paneni *et al.* 2013) – Akt: protein kinase B; FFA: free fatty acids; GLUT-4: glucose transporter 4; IKK-β: inhibitor of nuclear factor kappa-B kinase subunit beta; ILs: interleukins; IRS-1: insulin receptor substrate-1; JNK: c-Jun amino-terminal kinase; NFkB: nuclear factor-kappa B; PKC: protein kinase C; TLR: toll like receptor; TNFα: tumor necrosis factor α.

On a second hand, impaired insulin signaling induces dyslipidemia.

Free fatty acids are used in the liver to synthetize triglycerides. Elevation of free fatty acids promotes stabilization of apolipoprotein B (apoB) and as a consequence, promotes very light density lipoproteins (VLDL) production synthesis by the liver. In healthy conditions, apoB is degraded *via* insulin signaling, as well as VLDL degradation *via* lipoprotein lipase. VLDL is metabolized into remnant lipoproteins and light density lipoproteins LDL, both of which are atherogenic (Lewis and Steiner 1996). HDL normally possess anti-atherogenic properties, as it reverses the cholesterol transport from blood to the liver for excretion, on top of other protective activities such as antioxidative, anti-inflammatory, antithrombotic and vasodilatory actions (Assmann and Nofer 2003).

Under insulin resistance, hypertriglyceridemia and decreased activity of lipoprotein lipase induce a raise in HDL triglyceride content. In parallel to deterioration of LDL and VLDL metabolism, hypertriglyceridemia also induces an increase in HDL triglyceride content by an elevation of cholesterol ester transport protein (CETP) activity that exchanges the triglycerides from VLDL to HDL, and the cholesteryl esthers from HDL to VLDL (Le Goff *et al.* 2004). As a result, plasma HDL levels are decreased, as they are better degraded by hepatic lipase when associated to triglycerides. These mechanisms result in increased production of atherogenic particles, and decreased protective effects of HDL, which constitutes one of the feature of metabolic syndrome and type 2 diabetes (Lamarche *et al.* 1999). They are closely associated with oxidative stress and endothelial dysfunction, reinforcing the proinflammatory nature of macrovascular atherosclerotic disease (Kaur 2014).

The fact that abdominal obesity leads to increase in adipocytokines levels is a feature of metabolic syndrome. It results in adipose tissue inflammation that favors atherothrombosis and atherosclerosis, insulin resistance, liver steatosis and pancreatic β-cell function impairment (Kaur 2014).

On top of these mechanisms, adiponectin, recognized as a regulator of insulin sensitivity, food intake, and anti-inflammatory hormone (Liu and Liu 2009), is negatively associated to body weight and waist circumference (Xydakis *et al.* 2004) and low levels can be predictive for cardiovascular event (Pischon *et al.* 2004).

Another important contributing hormone is leptin, an adipokine responsible for satiety (Lau *et al.* 2005). Usually, its circulating levels increase in obese patients that progressively develop leptin resistance, as for insulin resistance. As a result, high plasma concentration cannot suppress the hunger anymore, *via* the leptin receptors located in the hypothalamus (Hutley and Prins 2005). Leptin has also been described as a hypertensive agent, through the sympathetic nervous system (Carlyle *et al.* 2002), and its action in increasing peripheral vascular resistance (Shirasaka *et al.* 2003).

It has also been reported that over synthesis of glucocorticoid (mainly cortisol) under chronic stress may lead to insulin hypersecretion, increase in body and visceral fat mass, and decrease in muscle mass, resulting in dyslipidemia, hypertension, and type 2 diabetes, in individuals with a genetic predisposition (Chrousos 2009).

All these mechanisms contribute to permanent inflammation that favors development and maintenance of insulin resistance in the body. Consequently, insulin resistance induce hyperglycemia responsible for micro and microvasculature defects that we will describe in the next section, leading to cardio-renal dysfunction, chronic diseases and finally can cause death (cardiovascular disease and chronic kidney disease).

1.2.3 Micro and macrovascular complications of hyperglycemia

Among several consequences of hyperglycemia, a major impact effect of metabolic syndrome with type 2 diabetes is the damage of blood vessels that will be described in this section. Impact on blood vessels (structure and function), triggers several pathologies such as diabetic retinopathy, glaucoma, cataracts, diabetic neuropathy, cardiovascular diseases, sexual dysfunction and diabetic nephropathy (Cade 2008).

The endothelium is a cell layer that surrounds the vascular lumen. This monolayer is necessary to maintain vascular homeostasis acting as endocrine, paracrine and autocrine organ (Vallance 2001; Bonetti *et al.* 2003). Its functions include regulation of vessel integrity, vascular growth and remodeling, tissue growth and metabolism, immune responses, cell adhesion, angiogenesis, hemostasis and vascular permeability.

As a result, endothelium controls tissue perfusion and inflammatory responses and regulates blood viscosity (Félétou and Vanhoutte 2006; Moncada and Higgs 2006; Sena *et al.* 2013).

Vasodilation and vasoconstriction are accurately balanced by endothelium, as well as proliferation of smooth muscle cells, fibrinolysis, thrombogenesis and adhesion and aggregation of platelets. This balance is very fragile and any disturbance has a major impact on vascular function and structure that can cause dramatic consequences on the organs function, like heart and kidney (Sena *et al.* 2013).

As displayed in Figure 4, the combination of hyperglycemia, high lipid levels and insulin resistance that characterizes metabolic syndrome, negatively impacts blood vessel structure and function through several mechanisms such as oxidative stress, activation of receptors for advanced glycation end product (RAGE) and PKC activation. The concept of glycemic continuum, *i.e.* the sequence of pre-diabetes, diabetes and cardiovascular risk shows that low levels of glycemia that are still under the threshold of overt diabetes already have negative effects on the vessels, and more particularly on the endothelium (Wei *et al.* 1998; Coutinho *et al.* 1999; Bartnik and Cosentino 2009; Rutter and Nesto 2011).

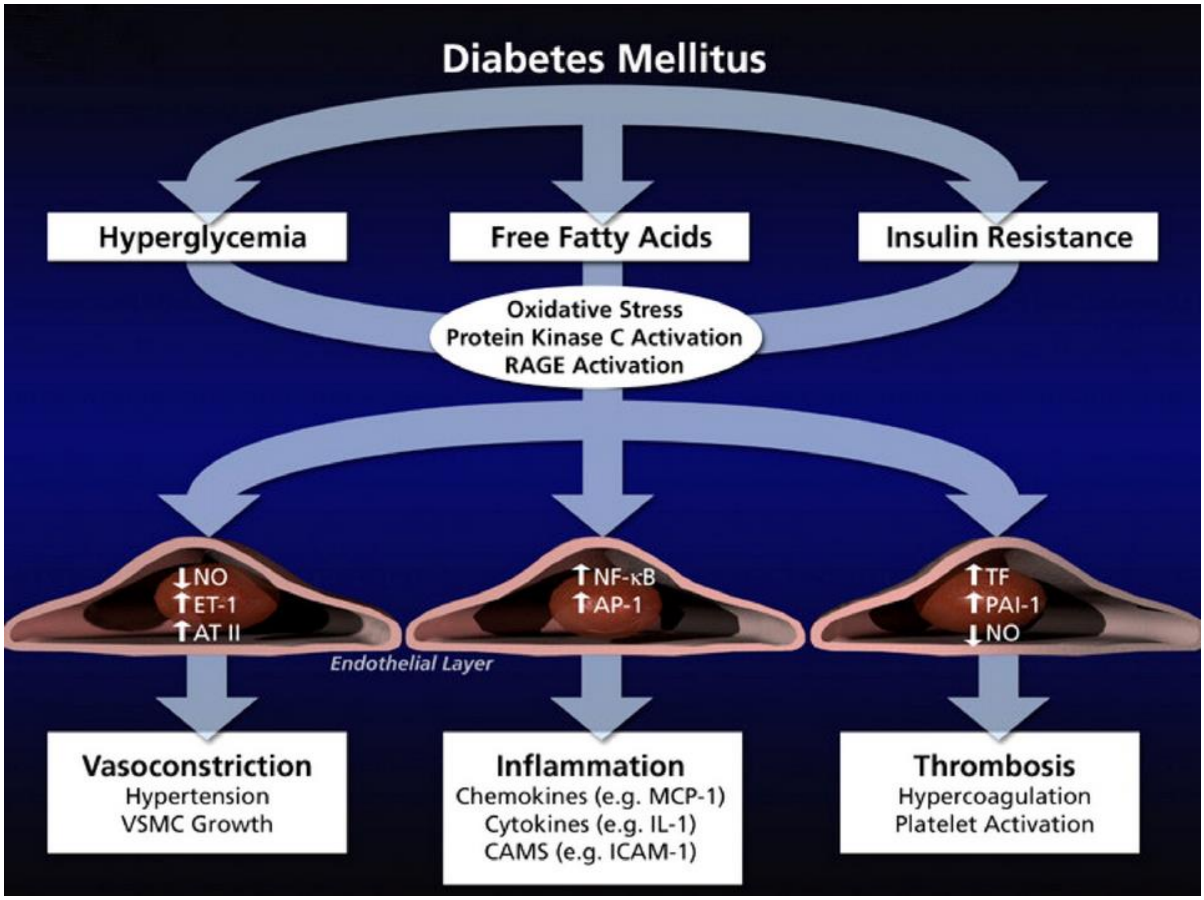

Figure 4 Alteration of endothelial function in the context of type 2 diabetes.

From (Creager *et al.* 2003). Schematic representation of cellular mechanisms triggered by hyperglycemia, elevated free fatty acids or insulin resistance, alone or in combination. These insults include increased oxidative stress, disturbances of intracellular signal transduction (such as activation of PKC) and activation of Receptor for Advanced Glycation Endproducts (RAGE). The consequences are decreased availability of nitric oxide (NO), increased production of endothelin (ET-1), activation of transcription factors such as NF-κB and activator protein-1 (AP-1), and increased production of pro-thrombotic factors such as tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1).

In the context of hyperglycemia and insulin resistance, endothelial dysfunction is then a consequence of enhanced overproduction and accumulation of reactive oxygen species (ROS) by mitochondria. This phenomenon is considered as the main link between hyperglycemia and diabetic vascular defects (Nishikawa *et al.* 2000; Creager *et al.* 2003).

High glycaemia activates PKC that induces alterations in cellular permeability, inflammation, angiogenesis, cell growth, extracellular matrix expansion, and apoptosis at the level of the endothelium (Geraldes and King 2010). Four main mechanisms of endothelial cellular damages have been described (Figure 5):

a- Superoxide anion (O_2^-) is produced *via* NADPH oxidase (Inoguchi *et al.* 2000) and, with nitric oxide (NO), induces the formation of oxidative agent peroxynitrite (ONOO−) that leads to nitration (introduction of a nitro group into an aromatic chemical compound, generally in tyrosine) of endothelial NO synthase (eNOS) and antioxidant enzymes (Hink *et al.* 2001). Moreover, PKCβ2 has been recently shown to phosphorylate $p66^{Shc}$, which causes an accumulation of ROS in the mitochondria (Cosentino *et al.* 2008; Paneni *et al.* 2012) and which was reported to enhance the NADPH activity, overall leading to production of ROS (Tomilov *et al.* 2010; Shi *et al.* 2011)

b- PKC activation enhances eNOS activity but as NO is also used as a reactive to produce ONOO−, as described above (Cosentino *et al.* 1997; Du *et al.* 2001; Alp and Channon 2004; Geraldes and King 2010), this leads to decrease NO levels. The lack of NO coupled to activation of PKC causes synthesis of ET-1 and prostanoids like thromboxane A2 (TA2), which are vasoconstrictors and inducers of platelet aggregation (Hink *et al.* 2001; Geraldes and King 2010).

c- Vascular inflammation is also triggered by PKC-dependent ROS production *via* NF-kB pathway, that induces elevation of monocyte chemo-attractant protein-1 (MCP-1), and adhesion proteins like vascular cell adhesion molecule-1 (VCAM-1) and intracellular cell adhesion molecule-1 (ICAM-1) (Kouroedov *et al.* 2004; Giacco and Brownlee 2010). As a result, activated macrophages produce cytokines that maintain the inflammatory status, and participate to atherosclerotic process by the formation of foam cells.

d- Methylglyoxal and advanced glycation end products (AGEs) synthesis are also a result of ROS production and are known to be highly involved in the process of endothelial dysfunction by increasing the production of ROS (American Diabetes Association 2013) (Brouwers *et al.* 2010; Yan *et al.* 2010; Sena *et al.* 2012).

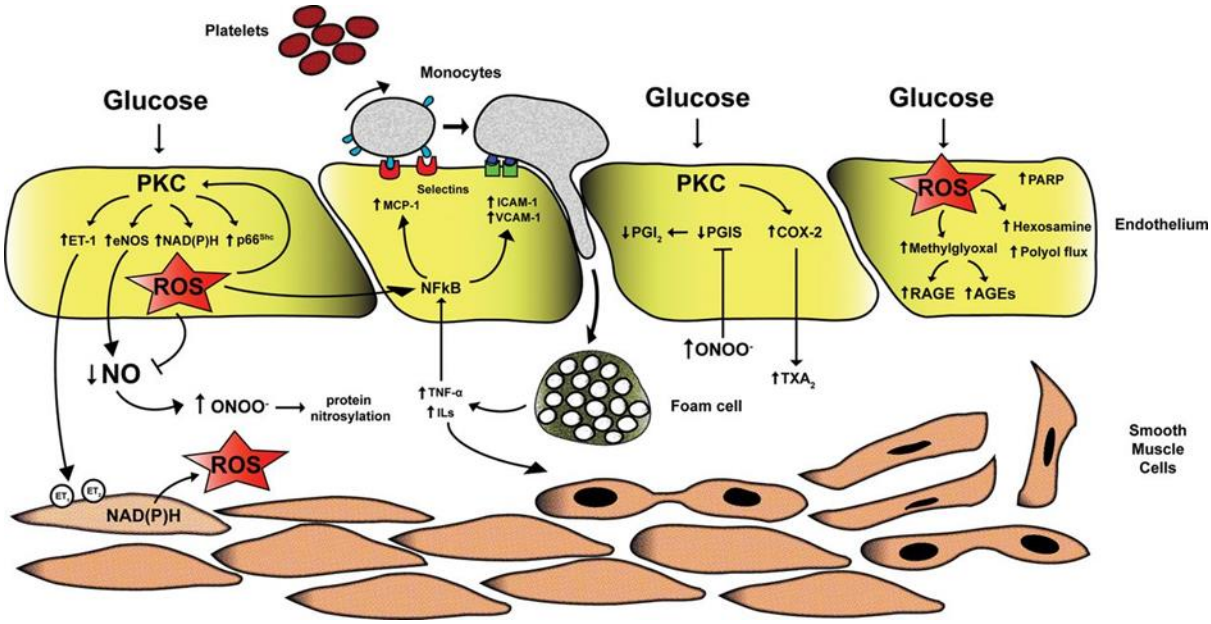

Figure 5 Mechanisms of hyperglycemia-induced vascular damage.

From (Paneni *et al.* 2013) – AGEs: advanced glycation end products; COX-2: cyclooxygenase-2; eNOS: endothelial nitric oxide synthase ; ET-1: endothelin-1; ICAM-1: intracellular cell adhesion molecule-1; ILs: interleukins; MCP-1: monocyte chemo-attractant protein-1; NF-ƙB: nuclear factor kappa binding; NO: nitrogen monoxide; $ONOO^-$: peroxynitrite; PARP: poly ADP ribose polymerase; PGI_2: prostacyclin; PGIS: prostacyclin synthase; PKC: protein kinase C; RAGE: receptor for advanced glycation endproducts; ROS: reactive oxygen species; TNFα: tumor necrosis factor α; TXA2: thromboxane A2; VCAM-1: vascular cell adhesion molecule-1.

Glycation was discovered by Maillard and consists in the chemical (non-enzymatic) reaction of reducing sugars with amino groups (Maillard 1912). Glycation generally impairs the function of biologic molecules, in contrast to enzymatic glycosylation of proteins, which is physiological. As an example, hemoglobin glycation leads to the formation of glycated hemoglobin (HbA1c). It results from

the formation of an adduct between glucose and the valine amino group of the β chain of hemoglobin. The determination of HbA1c in diabetes patients is a reliable indicator for monitoring glycemic control in diabetes patients (Wautier and Schmidt 2004). HbA1c is a marker used to follow the long term effects of a treatment on glycemia, as it integrates longer modifications than glycemia.

The initial glycation reaction is followed by a cascade of chemical reactions resulting in the formation of intermediate products (Schiff base, Amadori and Maillard products) and finally to a variety of derivatives named AGEs.

Moreover, AGEs have been detected in atherosclerotic plaques from diabetic patients showing that AGEs are closely linked to plaques formation (Niwa *et al.* 1997). Atherosclerosis consists in deposition of aggregates on arterial walls, and can be responsible for vessel occlusion and myocardial infarction (when plaques rupture happens). In atherogenesis, AGEs can bind arterial collagens that can trap plasma proteins or lipoproteins like LDL, to participate to atheroma formation in diabetes (Brownlee *et al.* 1985). Another important point for the atherosclerotic process is proliferation of smooth muscle cells. This cell proliferation is promoted by cytokines like insulin-like growth factor-I and platelet-derived growth factor whose secretion is promoted by AGEs as well (Ahmed 2005). Moreover, the RAGE can bind several ligands like AGEs, and is expressed on several cell types such as endothelial cells, monocytes and vascular smooth muscle cells (Kilhovd *et al.* 1999; Schmidt *et al.* 1999). The binding of ligands to RAGE have been reported to induce inflammatory responses in several chronic diseases including atherosclerosis (Naka *et al.* 2004).

One other mechanism that is believed to induce damage to vessels is linked to Aldose reductase. Aldose reductase is the initial enzyme in the intracellular polyol pathway that converts glucose into sorbitol. Hyperglycemia increases the activity of the polyol pathway. As a result, sorbitol accumulates in cells and that could be responsible for the development of diabetic microvascular complications by creating an osmotic stress. It has been shown in preclinical studies that sorbitol accumulation has

been linked to micro-aneurysm formation, thickening of basement membranes, and loss of pericytes (Gabbay 1975; Fong *et al.* 2004).

On top of these mechanisms, platelet aggregation is a consequence of insulin resistance as well. Indeed, lack of insulin signaling in platelets also impairs the IRS1/PI3K pathway resulting in Ca^{2+} accumulation in the cytosol necessary for platelet aggregation (Paneni *et al.* 2013). This phenomenon coupled with impaired fibrinolysis, mostly due to elevated levels of PAI-1, is a feature of type 2 diabetes (Collier *et al.* 1992; McGill *et al.* 1994). Moreover, elevated levels of coagulation activation markers, including thrombin-antithrombin complexes, have been found in patients with type 2 diabetes (Davì *et al.* 1992; Nagai 1994; López *et al.* 1999). Overall, these mechanisms favor hypercoagulable state and atherosclerosis.

To summarize, three main defects occur due to hyperglycemia, *via* ROS production: vasoconstriction, vascular inflammation and atherosclerosis. These three phenomena can contribute to development of hypertension, chronic inflammation and thrombosis.

1.3 HYPERTENSION

Hypertension is one of the main defects that characterize the metabolic syndrome, and it has been reported that metabolic syndrome is present in up to one third of the hypertensive patients (Cuspidi *et al.* 2004; Schillaci *et al.* 2004). The IDF defined blood pressure as a risk factor from 130 mm Hg of systolic blood pressure or diastolic blood pressure from 85 mm Hg (IDF 2005) (Table 1). In the metabolic syndrome, visceral obesity has been proposed to be the main trigger of hypertension. As stated previously, leptin, TNF-α, (IL-6), angiotensinogen, and free fatty acids are overproduced with fat accumulation. These entities also favor the development of hypertension, on top of the development of insulin resistance (Katagiri *et al.* 2007). Insulin has also been shown to induce renal

sodium reabsorption, and this anti-natriuretic effect seems to be exacerbated in an insulin resistant state, which can explain the link between hyperinsulinemia and development of hypertension (Sechi 1999). Other studies have shown that insulin induces production of ET-1, which as stated before is a potent vasoconstrictor that also favors hypertension (Sarafidis and Bakris 2007). In a previous section we described the interdependence between insulin resistance and oxidative stress and decrease of NO, which respectively induce sodium retention and decrease the vasodilation capacity of the vessels. Together, these features favor the onset of hypertension.

Abdominal obesity has also been involved in the overactivity of 2 main systems that enhance the elevation of blood pressure: the renin angiotensin aldosterone system and the sympathetic nervous system.

On one hand, insulin resistance, in combination with hyperinsulinemia, hyperleptinemia and obesity have been shown to induce activation of sympathetic nervous system. That activation induces sodium reabsorption by the renal proximal tubules which induce increase in volemia, and increase cardiac output. Moreover, obese people have impaired renal-pressure natriuresis, explaining the fact that these people need an increased blood pressure to maintain the sodium homeostasis (Hall 1997).

Leptin is also linked to sympathetic activity, as leptin has been shown to increase its activity through the ventromedial and dorsomedial hypothalamus, and consequently increase blood pressure (Carlyle *et al.* 2002; Marsh *et al.* 2003). Obstructive sleep apnea has been also related to an increased prevalence of metabolic syndrome, along with enhancement of sympathetic activity (Wolk *et al.* 2003; Coughlin *et al.* 2004), reinforcing its hypertensive action.

On the other hand, hyperinsulinemia coupled to hyperglycemia have been reported to activate key components of the Renin Angiotensin Aldosterone System (RAAS), such as angiotensinogen, angiotensin II (AngII), and its receptor isoform AT1, that are all involved in hypertensive process, *via* sodium retention and promotion of insulin resistance (Malhotra *et al.* 2001). RAAS will be further

described in the next section. More recently, it has been reported that AngII can trigger aldosterone synthesis by adipocytes, meaning that obese patients are more responsive to AngII as compared to fit individuals (Briones *et al.* 2012) (Figure 6).

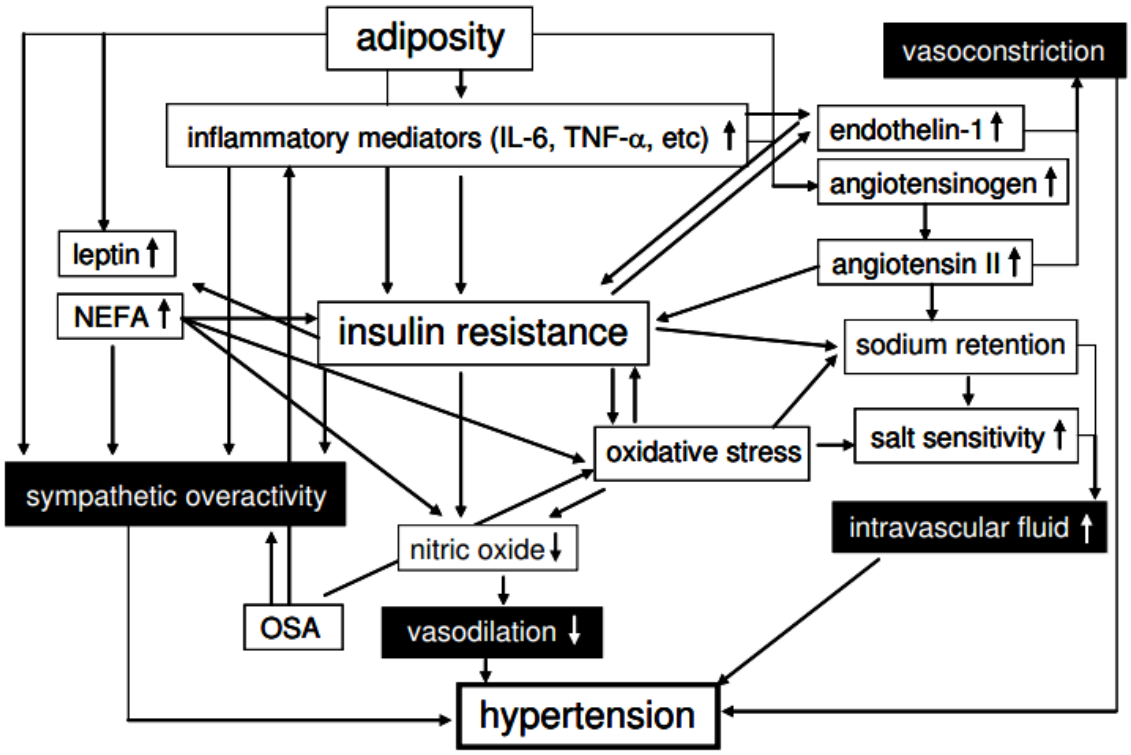

Figure 6 Proposed mechanisms responsible for hypertension onset in Metabolic Syndrome.

From (Yanai et al. 2008) – IL6 : interleukin 6 ; NEFA: non esterified fatty acids (or free fatty acids); OSA: obstructive sleep apnea; TNF-α : tumor necrosis factor α.

Finally, adiposity is a trigger for inflammation, insulin resistance, enhancement of sympathetic system and RAAS. All these defects are closely linked and together favor the development of hypertension, via elevation of volemia, vasoconstriction and decrease in arterial vasodilation capabilities.

To summarize, diet, insulin resistance, inflammation, oxidative stress, hypertension, are the main components of the metabolic syndrome. These features are the same that induce chronic kidney disease and cardiovascular disease which are consequently closely linked to metabolic syndrome (Raimundo and Lopes 2011).

1.4 IMPACT OF METABOLIC SYNDROME ON KIDNEY AND HEART

Microvascular complications in metabolic syndrome can lead to three main disorders, diabetic retinopathy which appear to be the most common complication of diabetes and can lead to blindness, diabetic neuropathy for which chronic sensorimotor distal symmetric polyneuropathy is the most common form, and diabetic nephropathy that we will focus on in this chapter.

Macrovascular complication in metabolic syndrome leads to cardiovascular diseases, mainly induced by the atherosclerosis process (Fowler 2011) and/or hypertension and arterial stiffness (Cecelja and Chowienczyk 2012). We will focus here on the diabetic nephropathy and cardiovascular diseases.

1.4.1 Diabetic nephropathy, chronic kidney disease, end stage renal failure and metabolic syndrome

Persistent microalbuminuria is the main defect that characterizes early diabetic nephropathy. In a normal subject, kidneys do not excrete large molecules like albumin. As a result, finding albumin in the urine is a sign of damaged renal structure and function. Microalbuminuria is defined by an albumin excretion rate of 20-200 µg/min or 30-300 mg/24h, or a spot urine albumin to creatinine ratio of 30-300 mg/g (3.5-35 mg/mmol) in males and 20-200 mg/g (2.5-25 mg/mmol) in females. Over 500 mg/24h of proteinuria or albuminuria >300 mg/24h, overt diabetic nephropathy is diagnosed (Reutens and Atkins 2011).

The precise pathogenesis of microalbuminuria in metabolic syndrome is not known. However, microalbuminuria in metabolic syndrome is believed to reflect RAAS activation resulting in oxidant stress, inflammation, and endothelial cells injury (Gobal *et al.* 2011).

Diabetic nephropathy develops in about one third of people with type 2 diabetes. The global prevalence of microalbuminuria in diabetes is 39% (Rossing 2006).

The exact cause of diabetic nephropathy is still unknown, but cytokines activation, AGEs are thought to be involved through hyperglycemia trigger. Moreover, hypertension can also be a catalyzer for the onset of diabetic nephropathy. Indeed, the resulting shear stress is responsible for increased production of growth factors and extra cellular matrix proteins, that favor mesangial expansion and extra cellular matrix deposition in the glomeruli (Schena and Gesualdo 2005).

Hyperglycemia also increases the expression of matrix proteins and transforming growth factor-β (TGF-β) in the glomeruli. Together with TGF-β, vascular endothelial growth factor (VEGF) is thought to participate in the cellular hypertrophy and enhanced collagen synthesis and may induce the vascular changes observed in patients with diabetic nephropathy (Chiarelli *et al.* 2009; Rask-Madsen and King 2010). Genetics also seem to play an important role in the predisposition of diabetic nephropathy like African Americans or Hispanic population for example. A polymorphism in the gene for angiotensin-converting enzyme (ACE) is either predisposing and/or accelerating to nephropathy (Naresh *et al.* 2009). However, genetic markers still have to be identified, although the role of epigenetic factors like micro-RNAs have been involved in this predisposition to diabetic nephropathy development (Deshpande *et al.* 2013).

Currently, diabetic nephropathy is a major cause of chronic kidney disease worldwide. It is also one of the most significant long-term complications in terms of morbidity and mortality for individual patients with diabetes.

Three major histologic changes occur in the glomeruli of patients with diabetic nephropathy. First, mesangial expansion is induced by hyperglycemia, and eventually *via* increased matrix production or glycosylation of matrix proteins, then thickening of the glomerular basement membrane occurs, and finally glomerular sclerosis, caused by intraglomerular hypertension (Fioretto and Mauer 2007).

Chronic kidney disease is a condition in which the kidneys lose their ability to excrete waste and regulate volemia. Chronic kidney disease has been defined in 2002 by the US National Kidney Foundation Kidney Disease Outcomes Quality Initiative (KDOQI) clinical practice guidelines as a lowering in glomerular filtration rate (GFR; lower than 60 ml/min/1·73 m^2 for at least 3 months) (KDOQI 2002). In 2012, The kidney disease: improving global outcomes (KDIGO) has refined the classification of chronic kidney disease according to 5 grades of glomerular filtration rate and 3 grades of albuminuria as displayed in Figure 7. This classification is the updated one and replaces the former from KDOQI. This new guideline recommends eliminating the term "microalbuminuria," and instead stages A1, A2, and A3 for the degree of albuminuria. There is a much greater emphasis on using this new scheme of classification for the prognostication of cardiovascular endpoints as well as renal endpoints and mortality as the different colors indicate risk for various outcomes depending on the degree of albuminuria and the level of GFR (Astor *et al.* 2011; Stevens and Levin 2013).

Prognosis of CKD by GFR and Albuminuria Categories: KDIGO 2012

GFR categories (ml/min/ 1.73 m^2) Description and range				Persistent albuminuria categories Description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				<30 mg/g <3 mg/mmol	30-300 mg/g 3-30 mg/mmol	>300 mg/g >30 mg/mmol
	G1	Normal or high	≥90			
	G2	Mildly decreased	60-89			
	G3a	Mildly to moderately decreased	45-59			
	G3b	Moderately to severely decreased	30-44			
	G4	Severely decreased	15-29			
	G5	Kidney failure	<15			

Figure 7 Current chronic kidney disease nomenclature used by kidney disease: improving global outcomes (KDIGO).

Modified from (Stevens and Levin 2013) – CKD: chronic kidney disease; GFR: glomerular filtration rate; Green: low risk (if no other markers of kidney disease, no chronic kidney disease); Yellow: moderately increased risk; Orange: high risk; Red: very high risk.

The UK Prospective Diabetes Study (UKPDS), has reported that 38% of 4031 patients developed albuminuria, and 1449 (29%) of 5,032 patients developed renal impairment (GFR < 60 mL/min per 1.73 m^2) over a 15-year period (Retnakaran *et al.* 2006). Moreover, the Developing Education on Microalbuminuria for Awareness of renal and cardiovascular risk in Diabetes (DEMAND) study, in which data from 32208 type 2 diabetics from 33 countries were collected, reported that overall global

prevalence of micro- and albuminuria was 39% and 10% respectively, while GFR below 60 mL/min per 1.73 m^2 occurred in 22% of the 11573 patients with available data (Parving *et al.* 2006).

According to the KDIGO clinical practice guidelines, a rapid decline in renal function was defined as a sustained yearly decline in GFR of > 5 mL/min per 1.73 m^2. Associated risk factors in patients with type 2 diabetes include lifestyle and health behaviors, poor glycemic control, some biochemical abnormalities, ethnic/genetic and demographic factors, albuminuria and dyslipidemia. Diabetic patients with glomerular hyperfiltration, elevated serum cystatin-C levels, retinopathy or cardiac neuropathy are at increased risk of a rapid decline in GFR (Sheen and Sheu 2014).

Prevalence is estimated to be 8–16% worldwide and complications include cardiovascular mortality, kidney disease progression, and acute kidney injury, among others. Worldwide, type 2 diabetes is the most common cause of chronic kidney disease (Jha *et al.* 2013).

The disease can progress to end-stage renal disease, *i.e.* kidney failure, corresponding to grade 5 of chronic kidney disease. This occurs when kidney function is so impaired that renal replacement therapy, *i.e.* hemodialysis, peritoneal dialysis or kidney transplantation is required to maintain life.

1.4.2 Cardiovascular disease linked to chronic kidney disease and metabolic syndrome

Cardiovascular disease affects the heart and blood vessels and is composed of coronary heart disease, stroke, and peripheral artery disease due to atherosclerosis that may cause fatal complications. Cardiovascular disease is the most common cause of death in people with diabetes. Risk factors for developing cardiovascular complications exist, such as high blood pressure, high cholesterol, high blood glucose, *i.e.* the components of metabolic syndrome (Witteles and Fowler 2008; Battiprolu *et al.* 2010). There is a link between diabetic nephropathy and cardiovascular events. It has been reported that patients with chronic kidney disease have a strong risk of developing

cardiovascular disease (Go *et al.* 2004). However, as chronic kidney disease and cardiovascular diseases share some risk factors such as hypertension or type 2 diabetes, it is hard to conclude that kidney disease is responsible for cardiovascular diseases in a context of metabolic syndrome. A collaborative meta-analysis performed in 2010 in 105872 participants derived from the general population showed that albuminuria ≥1.1 mg/mmol (10 mg/g) and low GFR (<60 ml/min/1.73 m^2) were independently predictive of all-cause and cardiovascular mortality (Matsushita *et al.* 2010). In the UK Prospective Diabetes Study, a link was established between impaired renal function and cardiovascular mortality (Adler *et al.* 2003). The relationship between albuminuria and risk factor to develop a cardiovascular event was primarily studied in the diabetic population, but evidences seem to support that the association is true in the diabetic and non-diabetic populations as well. (Basi *et al.* 2008). Several studies suggest that the risk of adverse cardiovascular events along with albuminuria increase from microalbuminuria to proteinuria (Basi *et al.* 2008). The Heart Outcomes Prevention Evaluation study demonstrated that baseline microalbuminuria was predictive for cardiovascular events such as myocardial infarction, stroke and cardiovascular death, but also all-cause mortality and hospitalization for congestive heart failure in people with or without diabetes (Gerstein *et al.* 2001). In patients with type 2 diabetes the relative risk to develop a major cardiovascular event was 1.97 (95% CI 1.68–2.31) whereas those without had a relative risk of 1.61 (95% CI 1.36–1.90). The Irbesartan Diabetic Nephropathy Trial also reported that albuminuria was an important risk factor for cardiovascular events (Anavekar *et al.* 2004).

Cardiovascular disease is the leading cause of death in type 2 diabetes patients (Laakso 1999). Diabetes increases by 4 fold the risk to have a cardiovascular disease as compared to non-diabetic patient, by 5 fold the risk of myocardial infarction, with increased risk of congestive heart failure after a first myocardial infarction episode (Haffner *et al.* 1998; Malmberg *et al.* 2000). Mufunda *et al.* in 2006 have predicted that 80% of the diabetic patients would die from cardiovascular disease (Mufunda *et al.* 2006).

To summarize, the combination of hyperglycemia, insulin resistance, dyslipidemia, hypertension, and chronic inflammation can injure the vascular endothelium, leading to macrovascular complications and cardiovascular disease in people with type 2 diabetes (Beckman *et al.* 2002).

Moreover, diabetic patients can develop cardiomyopathy which can be independent from hypertension or atherosclerosis. Early, middle and late phases have been described for the development of cardiomyopathy: early and middle phases have been thought to represent physiological adaptation to the metabolic alterations of type 2 diabetes and the late phase involves degenerative changes in the myocardium that progressively become irreversible, leading to a pathological heart function (Fang *et al.* 2004) (Table 3). One link between metabolic syndrome and cardiomyopathy could for example be the protein FoxO1 (Forkhead box protein O1). In mice it has been shown that metabolic stress-induced activation of FoxO1 is central to the development of diabetic cardiomyopathy (Battiprolu *et al.* 2012). FoxO proteins are targets of insulin signaling through the PI3K/Akt pathway. FoxO proteins have been shown to be implicated in cell growth, metabolism and survival (Ferdous *et al.* 2010). Activation of FoxO transcription factors triggers signaling elements that control both insulin sensitivity and glucose metabolism (Ni *et al.* 2007; Tremblay and Giguere 2008). Moreover, in *db/db* mice and a high fat diet – type 2 diabetes mouse models, Battiprolu *et al.* have shown that FoxOs were persistently active in hearts, and that FoxO activation was required in mice that had a high fat diet-induced heart failure (Battiprolu *et al.* 2012).

Table 3 The natural history of cardiomyopathy.

From (Battiprolu *et al.* 2010) – LV: left ventricle.

Phase	Molecular and cellular events	Alterations in structure and morphology	Myocardial performance
Early	• Metabolic disturbances: hyperglycemia, increased circulating free fatty acids, insulin resistance • Altered Ca^{2+} homeostasis • Endothelial dysfunction	• Insignificant changes in myocardial structure: normal LV dimensions, wall thickness, and mass	• Impaired diastolic compliance with normal systolic function, or no obvious functional changes
Middle	• Cardiomyocyte injury, apoptosis, necrosis • Activation of cardiac fibroblasts leading to myocardial fibrosis	• Minor changes in structure: slightly increased heart mass, wall thickness or size. • Cardiomyocyte hypertrophy • Insignificant myocardial vascular changes	• Significant changes in diastolic and systolic function
Late	• Hypertension • Coronary artery disease • Microangiopathy • Cardiac autonomic neuropathy	• Significant changes in structure: increased heart size, wall thickness and mass. • Myocardial microvascular disease	• Abnormal diastolic and systolic function

1.5 DESCRIPTION AND INVOLVEMENT OF RENIN ANGIOTENSIN ALDOSTERONE SYSTEM IN METABOLIC SYNDROME

RAAS dysregulation is highly involved in the onset of hypertension in the context of metabolic syndrome. As a result this is the first targeted system for the treatment of hypertension in the metabolic syndrome (See Section 1.6). For this reason, we will focus and describe RAAS in the following section.

1.5.1 Systemic RAAS

The main function of RAAS is to maintain homeostasis of blood pressure, extracellular volume and tissue perfusion. Dysregulation of RAAS has notable consequences on blood pressure. RAAS is

constituted of a cascade of hormones for which synthesis is regulated by enzymes that are often pharmaceutical targets, as shown in Figure 8.

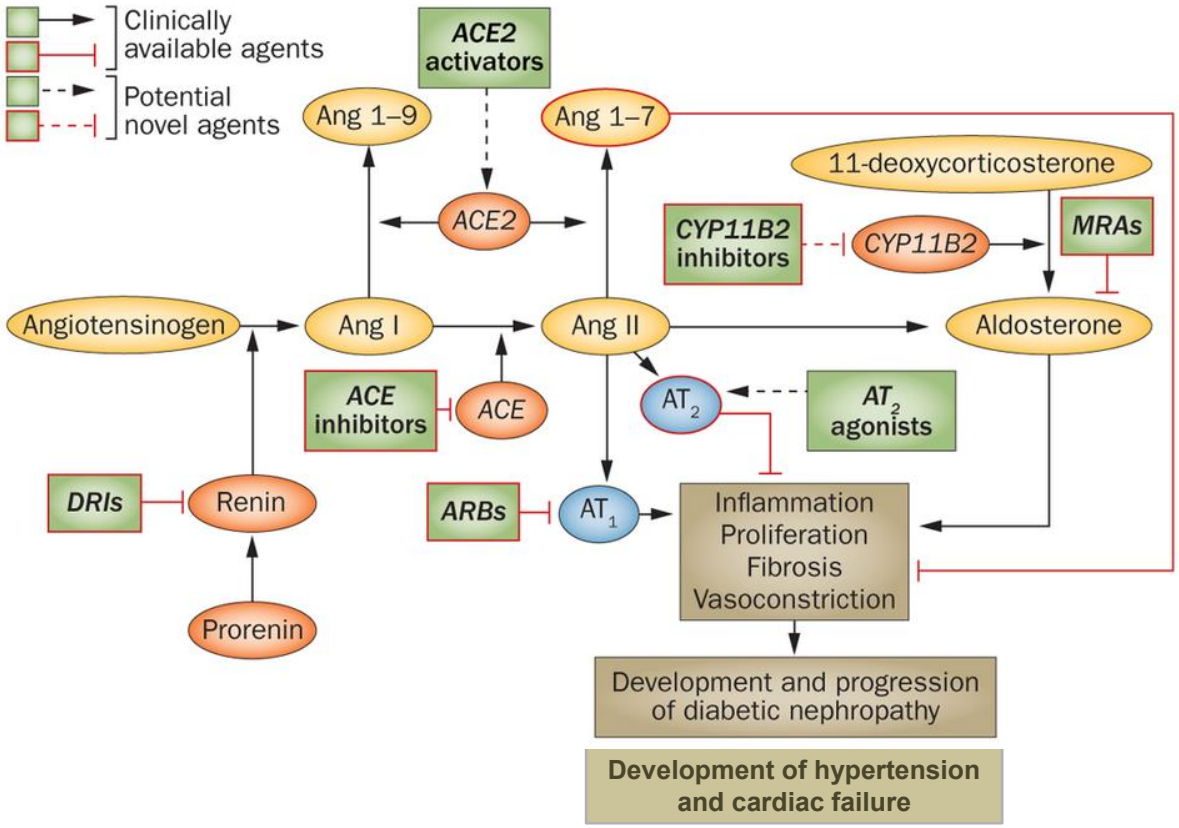

Figure 8 Current and potential targets for therapeutic interventions in the RAAS cascade.

Adapted from (Roscioni *et al.* 2014) ACE: angiotensin-converting enzyme; ACE2: angiotensin-converting enzyme 2; Ang I: angiotensin I; Ang II: angiotensin II; ARB: angiotensin-receptor blocker; AT1: type I angiotensin II receptor; AT2: type 2 angiotensin II receptor; CYP11B2: aldosterone synthase; DRI: direct renin inhibitor; MRA: mineralocorticoid receptor antagonist.

Prorenin is an aspartate protease produced in renal juxtaglomerular cells in response to decrease in volemia (Atlas 2007). Renin is produced from prorenin and is regulated by baroreceptor mechanism in the afferent renal arterioles, changes in NaCl concentration in the macula densa cells of the distal tubule, β1-adrenergic receptors from sympathetic system, and negative feedback mechanism from AngII (Brown 2006). Prorenin receptor (PRR) has been recently discovered and found to be

expressed in brain, heart, liver, brown adipose tissue, kidney and placenta. PRR can bind prorenin and renin with less affinity. Renin binding to PRR induces a change in biologically inert prorenin conformation into active form, *i.e.* with the same activity as renin. This constitutes an important discovery, as it can explain local AngII synthesis in some tissues that do not have the renin gene, like blood vessels or heart: this is called tissue RAAS that will be further developed in the next section Tissue RAAS and other sites of angiotensin synthesis (Section 1.5.2) (Nguyen *et al.* 2002; Danser 2010). The local synthetized AngII can act as a paracrine and autocrine hormone, independently regulated from the systemic RAAS (Kobori *et al.* 2007). In rats, non-proteolytic activation of prorenin has been shown to favor the development of spontaneous insulin resistance in type 2 diabetic rats through local RAAS activation in skeletal muscle and adipose tissues (Rafiq *et al.* 2011). In other terms, PRR is locally responsible for the production of AngII which has been described to enhance insulin resistance at the level of adipose tissue, pancreas, and muscles (see Section 1.5.3, Link between RAAS, insulin resistance and metabolic syndrome). Moreover, the binding of prorenin on PRR also triggers signal transduction independently from AngII pathway (Nguyen *et al.* 2002; Zhang *et al.* 2008) involving mitogen-activated protein kinase (MAPK), responsible for production of TGF-β1, a pro-inflammatory cytokine (see Section 1.5.2.2, Kidney RAAS). By this other mechanism, PRR could be linked to development of insulin resistance. Finally, PRR seems to be linked to Vacuolar H^+-ATPases, responsible for the control of cellular and intracellular vesicles pH, and that could be linked to insulin resistance as well (Balakumar and Jagadeesh 2011; Ichihara and Kinouchi 2011) (Figure 9).

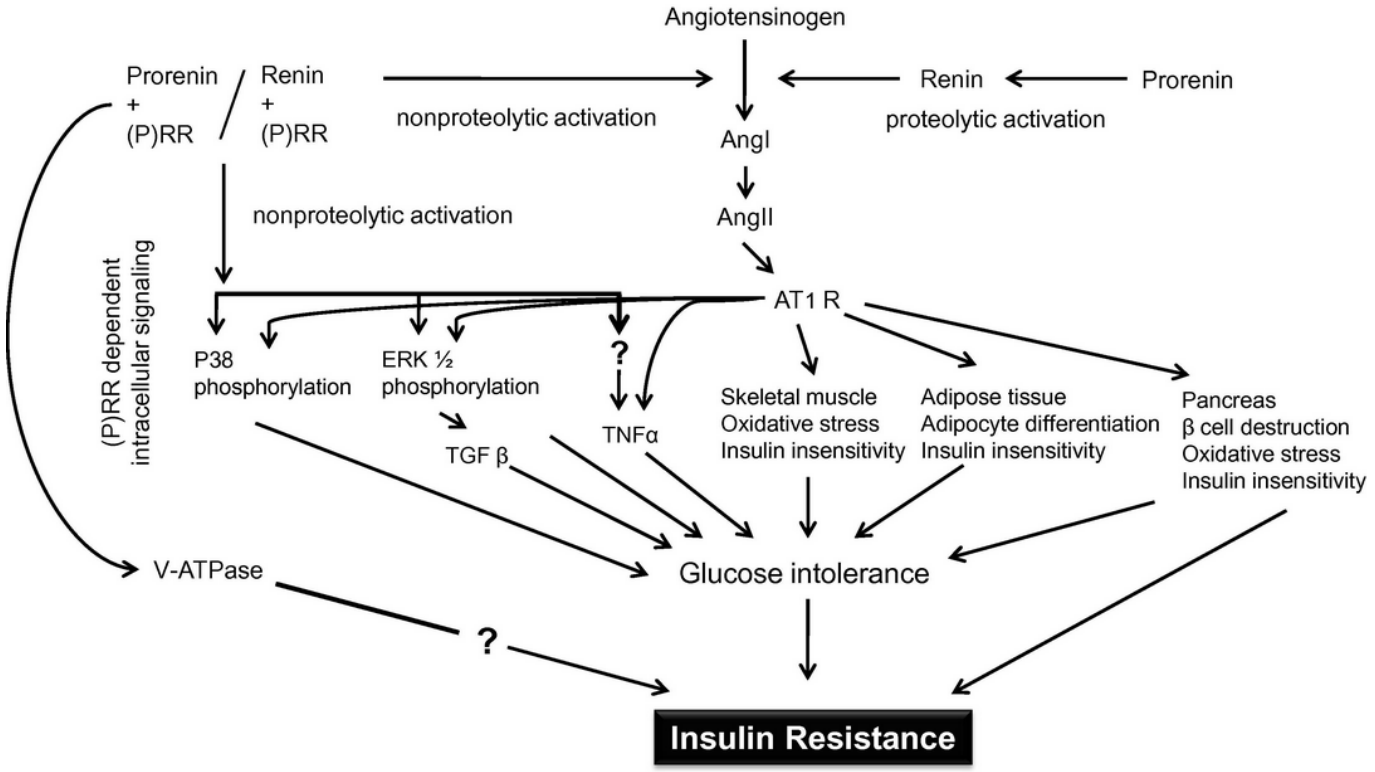

Figure 9 Schematic diagram showing the possible involvement of prorenin dependent system in the development of insulin resistance through angiotensin II-dependent and -independent pathways.

From (Rafiq *et al.* 2013) – AT1R: angiotensin II receptor 1; ERK: extracellular-signal regulated kinase; (P)RR: prorenin receptor; TGF-β: transforming growth factor β; TNF-α: tumor necrosis factor α; V-ATPase: vacuolar H^+-ATPase.

Renin is responsible for the cleavage of angiotensinogen, mainly produced in the liver and found in constant plasma concentration in a normal status, into angiotensin I (AngI) which is biologically inactive.

AngI is a decapeptide hydrolyzed in the octapeptide AngII by the Angiotensin Converting Enzyme (ACE), mainly located in the membrane of endothelial cells.

AngII is the key player of the RAAS, known as a potent vasoconstrictor mainly acting *via* its receptor AT1 to produce several effects such as vasoconstriction, inhibition of renin release, stimulation of aldosterone synthesis and release in the adrenal *zona glomerulosa.* AngII increases blood pressure by several mechanisms (Harrison-Bernard 2009):

- AngII causes vasoconstriction of systemic and renal arterioles, thus increasing total peripheral resistance and blood pressure,
- It also triggers sympathetic output to the heart and vasculature, inducing an elevation of cardiac output and total peripheral resistance, thus increasing blood pressure,
- AngII induces an increased release vasopressin from the pituitary gland, which increases fluid retention in the renal collecting duct to maintain volume homeostasis,
- AngII stimulates thirst, which also raises blood pressure,
- Finally AngII increases aldosterone release from the adrenal cortex, which increases sodium and fluid reabsorption in the distal nephron.

Three other AngII receptors have been identified. AT2 is mainly expressed during prenatal life, and is also known to be modulated by pathological states associated with tissue remodeling or inflammation such as hypertension, atherosclerosis, and myocardial infarction (Lemarié and Schiffrin 2010), and induce a vasodilation and antiproliferative mechanisms at the level of vascular smooth muscle cellular layer (Carey and Siragy 2003). In response to AngII, inflammatory cytokines (IL-1, TNFα) or glucocorticoids, angiotensinogen plasma levels can be increased (Morgan *et al.* 1996). AT3's function is not known and AT4 might be involved in PAI-1 release by AngII signaling (Stanton 2003). Finally, AngII favors synthesis of inflammatory, fibrogenic and angiogenic components, promotes ROS formation and interstitial fibrosis and hypertrophy, which can lead to organ damage (Sun 2010).

Other angiotensins have been identified although AngII is the most biologically active, and originate from the degradation of the N-terminus part of AngII by aminopeptidases. Angiotensin III (AngIII) is thought to act on the central nervous system to maintain tonic blood pressure and hypertension and to induce with AngII a synthesis and release of aldosterone. Angiotensin IV (AngIV) is believed to potentiate the effects of AngII on AT1 (Reudelhuber 2005). From the C-terminus of AngII, angiotensin

1-7 (Ang1-7) can be produced by a carboxypeptidase named ACE2 (Carey and Siragy 2003). Ang1-7 acts *via* Mas-receptor (Mas-R) to produce vasodilation and cardioprotective effects. Angiotensin 1-9 (Ang 1-9) can be produced from AngI by ACE2 as well, which is biologically inactive (Reudelhuber 2005).

Aldosterone is the last important hormone constituting the RAAS. Aldosterone is an antidiuretic steroid hormone synthetized in the zona glomerulosa of adrenal cortex. Aldosterone synthase's activity (or CYP11B2) is enhanced by AngII, and aldosterone is produced from its precursor 11-deoxycorticosterone (Clyne *et al.* 1997). Its main role is to enhance sodium reabsorption in the distal nephron of the kidney, mediated by the epithelial sodium channel, and as a result is a key regulator of extracellular volume. Moreover, aldosterone can negatively impact several organs in a context of inflammation, oxidative stress and high salt diet, particularly on heart, kidney and blood vessels. Aldosterone is involved in the development of renal fibrosis, podocyte injury and mesangial cell proliferation (Figure 10), by induction of ROS, inflammation and fibrosis, among others due to nuclear factor κB (NF-κB) induction or Serum and Glucocorticoid-regulated Kinase 1 (SGK1), both belonging to the Glucocorticoid pathway (Brown 2013). These effects negatively impact kidney function, by inducing pro-oxidative stress, triggering genotoxic effects and inflammatory pathways leading to fibrosis (Ritz and Tomaschitz 2013; Hargovan and Ferro 2014). It is also linked to cardiovascular system *via* its deleterious effect on endothelial function, induction of vascular fibrosis and cardiac hypertrophy (Briet and Schiffrin 2010).

In summary, RAAS dysregulation is central in the development of several major pathologies including arterial stiffness, endothelial dysfunction, hypertension, and progression towards chronic kidney disease and cardiovascular disease. All these features all together participate to the development of the metabolic syndrome. In other words, RAAS is highly involved in the metabolic syndrome (Mahmud and Feely 2004; Yki-Jarvinen and Westerbacka 2007; Milan *et al.* 2011; Targosz-Korecka *et al.* 2013; Underwood and Adler 2013).

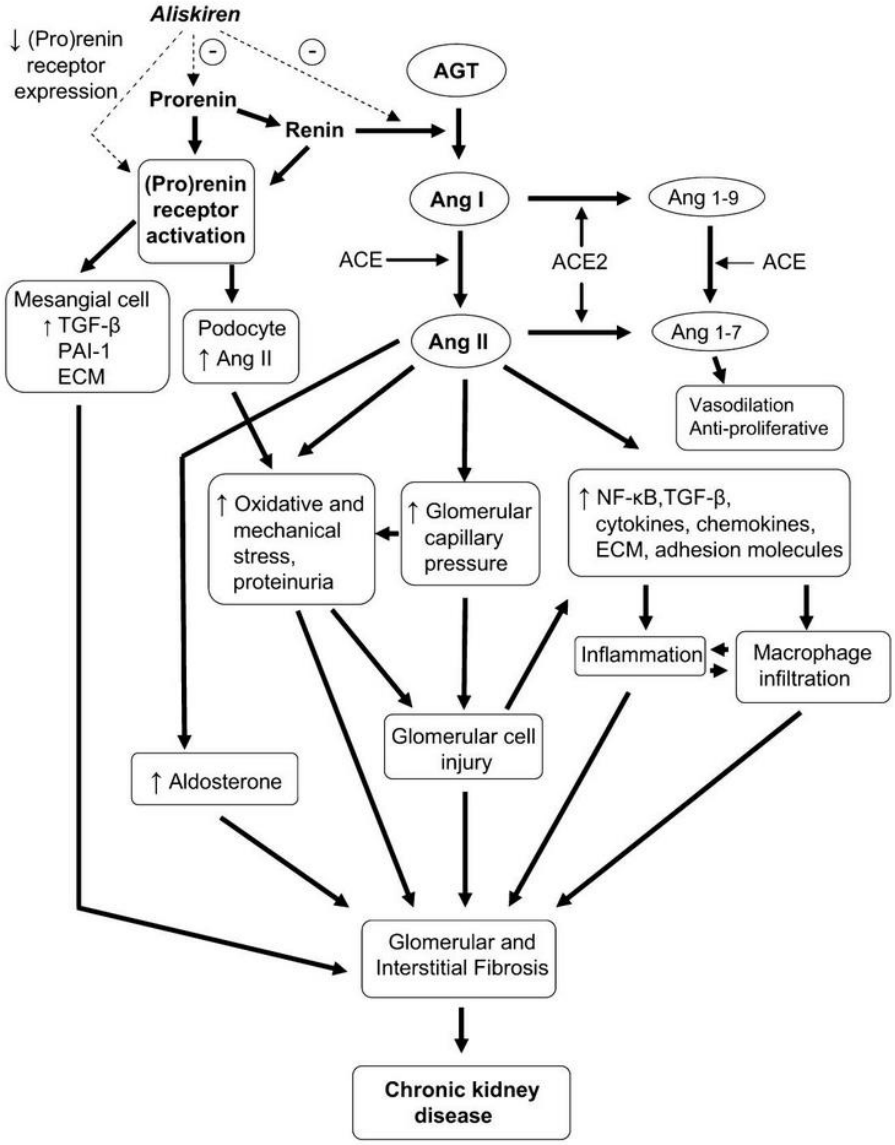

Figure 10 Multiple roles of the RAAS in the pathogenesis of chronic kidney disease.

From (Siragy and Carey 2010) — ECM: Extracellular matrix; NF-κB: nuclear factor-κB;TGF-β: transforming growth factor β.

1.5.2 Tissue RAAS and other sites of angiotensin synthesis

There is evidence of an existing tissue RAAS, meaning local synthesis of AngII by tissues. For example, tissue RAAS has been shown to exist in heart, blood vessels, kidney, adrenal gland or adipose tissue (Carey and Siragy 2003). It is reported that about 40% of the AngII levels found in the human kidney comes from non-ACE pathways (Hollenberg *et al.* 1998). The following sections will describe in more detail local RAAS located in the heart, kidney and adipose tissue. These local RAAS appear to act in a paracrine/autocrine manner and to regulate organ function. Local RAAS are involved in pathologic events associated with end-organ damage.

1.5.2.1 Heart RAAS

Circulating angiotensinogen and renin are thought to be taken up by cardiac cells (Danser *et al.* 1994), and to play their role by producing AngI, which is converted into AngII, as it is observed in the endocrine system. AngII induce myocyte hypertrophy on top of an indirect (*via* activation of noradrenaline release by sympathetic nerves) inotropic effect (Carey and Siragy 2003; Paul *et al.* 2006). In normal conditions, heart RAAS seems to play a role in cell growth control, proliferation, and reaction to myocardial stretch (Paul *et al.* 2006). In pathological conditions, as for the circulating RAAS, heart RAAS is believed to enhance ventricular hypertrophy mediated *via* AngII (Pagliaro and Penna 2005).

AngII plays a dual role in the heart since it stimulates scar formation in case of myocardial infarction for example, by inducing synthesis of inflammatory, fibrogenic and angiogenic components. On the other hand AngII promotes ROS formation which is deleterious for tissues. Furthermore, AngII promotes interstitial fibrosis and hypertrophy, which can lead to ventricular dysfunction (Sun 2010).

1.5.2.2 Kidney RAAS

Angiotensinogen is produced by epithelial cells of the proximal tubule and is secreted into the lumen. Renin is synthetized into the juxtaglomerular cells, while ACE is located on the proximal tubule brush border (Kobori *et al.* 2006; Ortiz-Melo and Spurney 2014). Under these conditions, AngII can be produced to maintain and regulate local perfusion and GFR, in a normal state (Carey and Siragy 2003).

In an abnormal state, e.g. under high prorenin concentration, PRR can be activated in mesangial cells leading to increase in profibrotic mediators such as PAI-1 and TGF-β, and extracellular matrix (ECM) components like collagen-1 and fibronectin (Huang *et al.* 2006), independently from AngII action. These mechanisms occur *via* mitogen-activated protein kinase and extracellular signal-regulated kinase (MAPK-ERK) pathway (Nguyen *et al.* 2002). This can lead, among other defects, to glomerulosclerosis (Huang *et al.* 2006) that contributes to chronic kidney disease progression.

Moreover, glomerular hypertension can occur if AngII is overproduced, that causes hemodynamic damages to endothelium, epithelium and mesangial cells (Anderson *et al.* 1986; Rüster and Wolf 2006). Non-hemodynamic stress can be produced by aldosterone and AngII *via* profibrotic factors, pro inflammatory factors, production of ROS (Rüster and Wolf 2006) (Figure 10).

1.5.2.3 Adipose tissue RAAS

Adipose tissue is known to play an endocrine role, *via* adipokines (Kershaw and Flier 2004). Actually, increase in adipokines level parallels fat accumulation and obesity. White adipose tissue is another site of AngII production as all the RAAS components are present, acting as autocrine, paracrine and even endocrine tissue (Karlsson *et al.* 1998; Cassis 2000).

AngII produced in white adipose tissue also induces secretion of leptin (Cassis *et al.* 2004) and decreases production of adiponectin (Ran *et al.* 2006) in rats. It has also been shown that weight loss

is associated with a decrease in both white adipose tissue and systemic RAAS activity in humans (Tuck *et al.* 1981; Engeli *et al.* 2005).

1.5.3 Link between RAAS, insulin resistance and metabolic syndrome

RAAS is known to play an essential role in the onset of obesity and insulin resistance, on top of hypertension, features that together constitute the metabolic syndrome (Alderman *et al.* 1991; Boustany *et al.* 2004; Schmieder *et al.* 2007).

1.5.3.1 AngII effects

AngII has multiple actions on several organs, as described above, *via* systemic RAAS and local RAAS. On top of his pro-inflammatory and fibrotic roles, AngII is thought to induce insulin resistance, through several mechanisms in many organs, especially skeletal muscle, adipose tissue and pancreas (Underwood and Adler 2013). In the skeletal muscle, AngII impacts glucose uptake by alteration of local blood flow (Henriksen and Prasannarong 2013; Muniyappa and Yavuz 2013) and by direct effect on insulin signaling by induction of IRS-1 phosphorylation, that results in decrease in GLUT-4 translocation (Velloso *et al.* 1996; Ogihara *et al.* 2002; Goossens *et al.* 2004; Manrique *et al.* 2009). Moreover, AngII induces ROS production *via* NADPH oxidase and NF-kB pathway in rat skeletal muscle cells, therefore participating to the onset of insulin resistance (Wei *et al.* 2006; Wei *et al.* 2008).

In the adipose tissue, as mentioned above, AngII affects leptin and adiponectin levels, involved in glucose homeostasis. Moreover, adipocyte size can be increased by AngII (Goossens 2012), leading to a decrease in lipid storage capacity due to a reduced buffering capacity (morphological change), and consequently to an increase in circulating lipids, production of adipokines and insulin resistance (Frayn 2001; Goossens 2008).

Finally, in the pancreas, AngII is the main representative of RAAS (Chappell *et al.* 1991). AngII impacts blood flow, plays a role in decreasing insulin secretion by overproduction of NO in the pancreas that results in insulin secretion dysfunction and induces synthesis of pro-inflammatory and pro-fibrotic molecules (Leung 2007; Yuan *et al.* 2010).

1.5.3.2 Aldosterone effects

As displayed in Figure 11, aldosterone, *via* mineralocorticoid receptor (MR) signaling, negatively impacts several organs such as heart, blood vessels or kidney. For example, in kidney, aldosterone is involved in glomerulosclerosis, tubulointerstitial inflammation and fibrosis. In the heart, it is involved in cardiovascular inflammation, fibrosis and hypertrophic remodeling. In liver, aldosterone has been shown to increase gluconeogenesis (Briet and Schiffrin 2011).

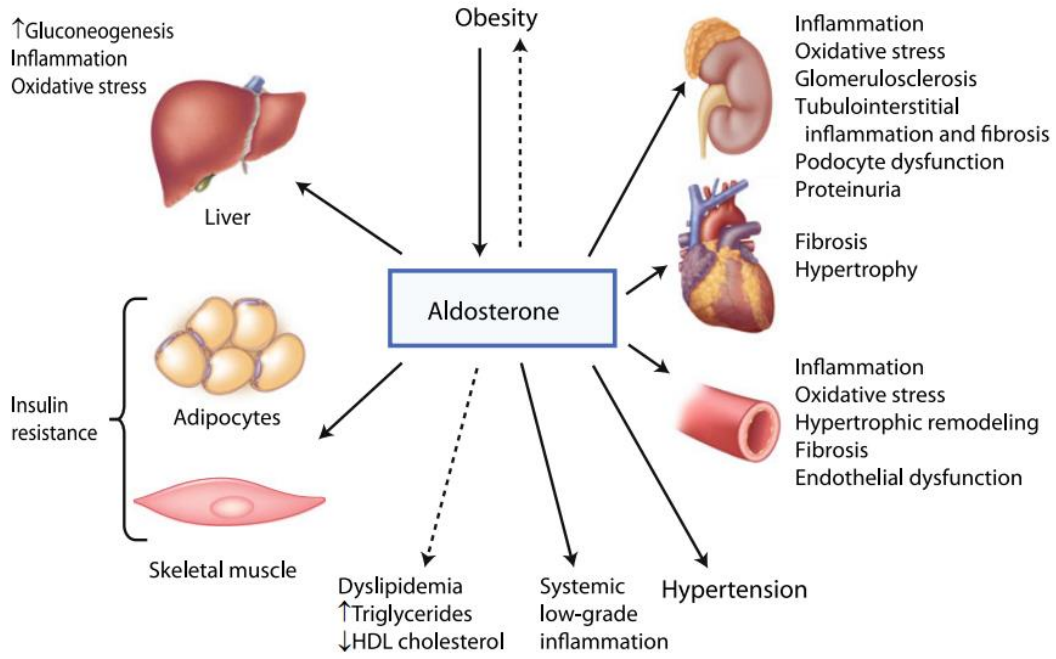

Figure 11 Effects of aldosterone on metabolic syndrome.

From (Briet and Schiffrin 2011).

Aldosterone acts *via* genomic effects *i.e.* mineralocorticoid receptors, which trigger a long process, and non-genomic effects (e.g. with no implication of transcription process) *via* MR or membrane-associated aldosterone receptor, involving transactivation of the epithelial growth-factor receptor (EGFR), which constitutes a quicker process (Perico *et al.* 2008; Dooley *et al.* 2012).

Figure 12 displays genomic and non-genomic actions of aldosterone. Genomic effects take place mainly in renal distal convoluted tubule *via* cytosolic mineralocorticoid receptors. Aldosterone induces transcription of pro-inflammatory genes such as TGF-β, MCP-1 or IL-6, pro-thrombotic factors such as PAI-1, enhances the NADPH oxidase activity inducing the production of ROS, on top of regulating Na+/K+ equilibrium.

In brain, cardiovascular system and skeletal muscle cells, aldosterone potentially acts *via* special cytosolic mineralocorticoid receptors in a non-genomic manner (Connell and Davies 2005). Aldosterone, and AngII *via* AT1, can both activate the Tyr kinase receptor EGFR that promotes the production of pro-inflammatory molecules *via* ERK pathway (Montezano and Touyz 2008; Lastra *et al.* 2010).

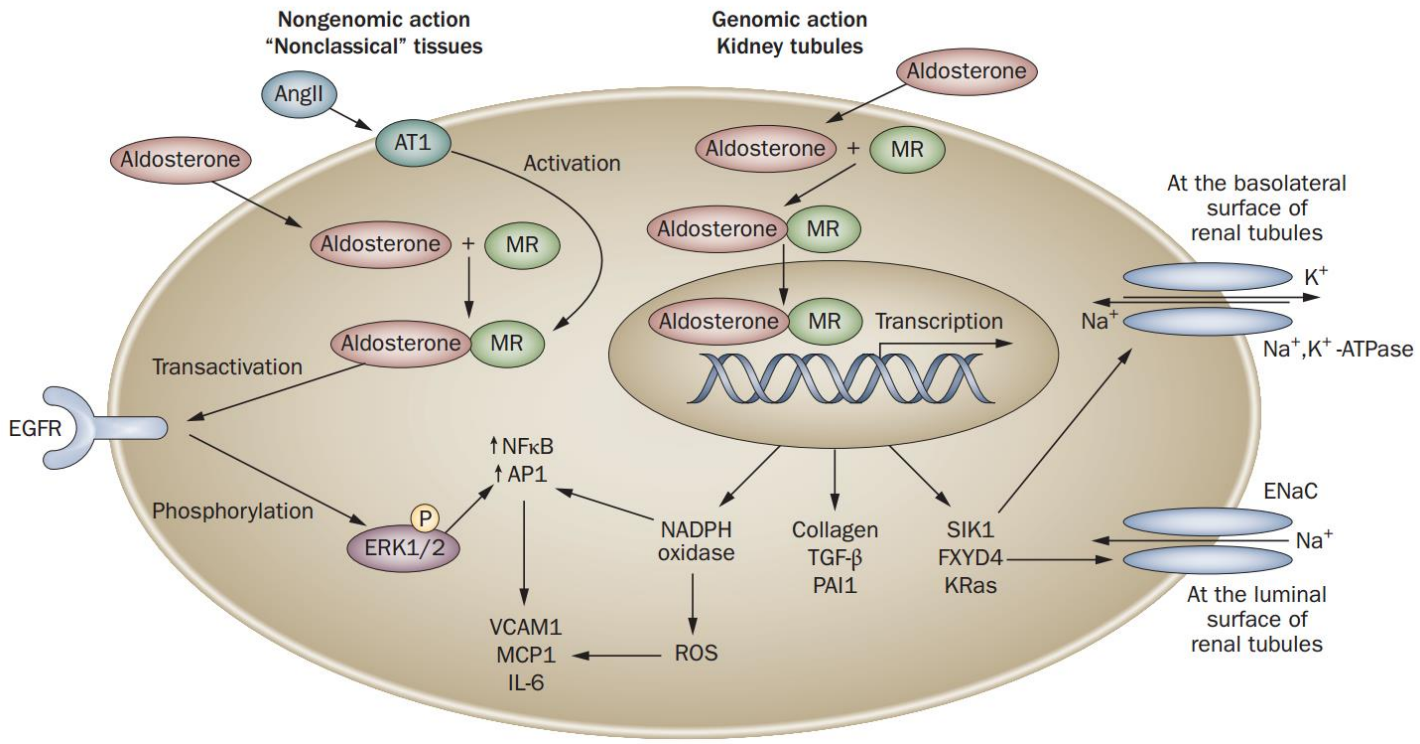

Figure 12 Genomic and non-genomic actions of aldosterone and interaction with the mineralocorticoid receptor.

From (Lastra *et al.* 2010).

To summarize, it is possible that ROS, inflammation-induced by RAAS, especially through AngII and increased aldosterone levels in a context of overweight, induce insulin resistance. These modifications seem to contribute to the onset of arterial stiffness, hypertension, renal and cardiac defects which can lead to cardiovascular disease and chronic kidney disease. Hemodynamic stress induced by hypertension, together with hormonal stress mainly provoked by increase in AngII and aldosterone, seem to contribute to glomerulosclerosis, renal fibrosis and renal inflammation, cardiac hypertrophy, cardiovascular inflammation and fibrosis.

1.5.4 Targeting the RAAS for treatment of metabolic syndrome

Several classes of drugs can reduce the RAAS activity as displayed in Figure 8 (see Section 1.5.1).

1.5.4.1 Direct Renin Inhibitors (DRIs; -kiren)

Renin is the starting point of the RAAS, explaining why it has been the most logical and first idea as a drug target in the RAAS, but the last to reach the market because of high specificity of the human enzymatic activity. DRIs were developed to reduce AngII and aldosterone synthesis and avoid their negative impacts on tissues and organ function. Pepstatin was the first renin inhibitor but showed a low efficacy and had poor solubility so it was not developed. Then analogue peptides of angiotensinogen were produced but were not very potent and were not stable. Peptide mimetics were developed and were shown to be potent but showed weak pharmacokinetic properties. Finally, after more than 30 years of research, Aliskiren, a non-peptidic inhibitor of renin was the first drug from this class on the market, approved only in 2007 by the Food and Drug Administration to treat hypertension (Jensen *et al.* 2008). It has been shown that in patients with diabetic nephropathy, proteinuria and blood pressure were reduced by Aliskiren (Persson *et al.* 2008). Aliskiren can be given alone or in association with other hypertensive agents. Aliskiren is well tolerated, but some adverse effects have been reported such as dizziness, fatigue and headaches. Moreover, as the bioavailability is relatively poor (2.6%), the administered doses are quite high, *i.e.* 150 to 300 mg daily (Vaidyanathan *et al.* 2008). As a result, this compound can accumulate in the colon, and provoke diarrhea. It has been reported that up to 300 mg daily, diarrhea events did not differ significantly from placebo, but when given at 600 mg daily, the incidence of diarrhea was significantly higher than that of placebo (11.4% vs 0.2%; $P < 0.001$) (Stanton *et al.* 2003).

1.5.4.2 ACE inhibitors (-pril)

These agents block the conversion of AngI to AngII, limiting its negative impact on blood pressure, inflammation, aldosterone production. Consequently, ACEi protect organs structure and function such as kidney, blood vessels and heart. This class of drugs is indicated in patients with hypertension, congestive heart failure, myocardial infarction, left ventricular dysfunction, diabetic nephropathy with proteinuria (Bicket 2002). Among others, ACE inhibitors have been shown to protect endothelial function by a decrease of ROS and prostanoid production (Mulder *et al.* 1995; Varin *et al.* 2000). Moreover, this class of drugs has shown antioxidant effects that may prevent and treat cardiovascular and renal alterations induced by AngII (Rugale *et al.* 2005). ACE inhibitors are well tolerated with the main adverse effect being cough (Sweitzer 2003). Heart Outcomes Prevention Evaluation (HOPE) and Microalbuminuria Cardiovascular and Renal Outcomes-HOPE (MICRO-HOPE) trials have shown good evidence of risk reduction for cardiovascular events in diabetic patients (The HOPE investigators 2000).

1.5.4.3 Angiotensin receptor blockers (ARBs; -sartan)

Complementary to ACE inhibition, ARBs block AngII effects mainly *via* AT1 receptors. ARBs are widely prescribed to hypertensive patients, patients suffering from kidney disease and heart failure (Terra 2003). ARBs and ACEi have complementary effects on angiotensin II inhibition since ACEi block AngII production, while ARBs block the non ACE-dependent AngII effects. In consequence, combination therapy with ACEi and ARBs can induce greater efficacy compared to monotherapies (Forclaz *et al.* 2003). Still, this synergistic effect of combination is still under debate, as the Veterans Affairs Nephropathy in Diabetes (VA NEPHRON-D) was stopped after 2.2 years only, for safety concerns, showing that patients with nephropathy should not be treated with this combination (Fried *et al.* 2013). Moreover, in the Ongoing Telmisartan Alone and in Combination with Ramipril Global Endpoint Trial (ONTARGET) trial, ramipril and telmisartan have shown the same efficacy as monotherapies in patients with vascular disease or high-risk diabetes patients, with more adverse

events without an increase in benefit when given in combination (Liebson and Amsterdam 2009). On the contrary, some other studies such as A Multicenter Trial Using Atacand and Zestril vs. Zestril to Evaluate the Effects on Lowering Blood Pressure (AMAZE) have shown beneficial effect of the combination between candesartan and lisinopril in hypertensive patients (Izzo *et al.* 2004). This shows that the combination can be beneficial in some populations and not in others, so that combination should be prescribed with care, in function of the patient disease.

ACEi and ARBs on the long run can stimulate pro-renin and renin production as a feedback mechanism, leading to an increase in AngII levels. In consequence the efficacy of these classes of compounds can decrease with time (Atlas 2007). Additionally, mono or combination therapy with ACEi and ARBs can trigger a compensatory elevation in plasma aldosterone called “aldosterone breakthrough” that counteracts the effects of ACEi or ARBs on organ protection (Bomback and Klemmer 2007). Aldosterone breakthrough can be explained by the non ACE-related AngII production pathways (Hollenberg *et al.* 1998) and by the fact that adrenocorticotropic hormone (ACTH) can also stimulate production of aldosterone (Spat and Hunyady 2004).

1.5.4.4 MR antagonists (MRAs; -one)

As described earlier, aldosterone is an antidiuretic hormone. Aldosterone plays a role in glomerulosclerosis, endothelial dysfunction and fibrosis via synthesis of pro-inflammatory factors and ROS. MRAs block the action of aldosterone on mineralocorticoid receptor. MRAs are prescribed in patients with hyperaldosteronism, essential hypertension, resistant hypertension, heart failure (Epstein and Calhoun 2011). In heart failure, MRAs can be given on top of ACE inhibitors or ARBs. The Randomized ALdactone Evaluation Study (RALES) evaluated the beneficial effect of spironolactone given on top of ACEi, diuretic, or digoxin, in patients with heart failure with reduced ejection fraction. This study showed a 30% reduction in all-cause mortality with spironolactone, with no significant increase of hyperkalemia or renal failure, evoking a protective effect on heart (Pitt *et al.* 1999). Then the Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival

Study (EPHESUS) showed that in patients with acute myocardial infarction complicated by left ventricular dysfunction and heart failure symptoms, eplerenone reduced mortality by 15% when given on top of ACEi, ARBs, diuretics or beta-blockers (Pitt *et al.* 2003). Finally, the Eplerenone in Mild Patients Hospitalization and Survival Study in Heart Failure (EMPHASIS-HF) has evaluated if treatment with eplerenone on top of standard of care (mainly beta-blockers) in patients with heart failure with reduced ejection fraction could improve survival rate. That study showed that eplerenone, as compared with placebo, reduced both the risk of death and the risk of hospitalization among patients with systolic heart failure and mild symptoms (Zannad *et al.* 2011). These studies definitely show the benefit of adding particularly eplerenone on top of standard of care to decrease mortality in patients with heart dysfunction.

Like for other existing kidney therapies, side-effects have been reported with MRAs. For example, sexual dysfunction has been reported for spironolactone, due to binding to progesterone and androgen receptors (Menard 2004). This side effect was not observed with eplerenone, which is more selective than spironolactone. Hyperkalemia can also be a consequence of MRAs, especially in a context of chronic kidney disease, and the use of loop diuretics (furosemide) or hydrochlorothiazide, can help to reduce kalemia (Epstein and Calhoun 2011).

1.5.4.5 Aldosterone synthase inhibitors (ASi)

Aldosterone synthesis is mainly controlled by regulation of transcription of the CYP11B2 gene, encoding for aldosterone synthase that catalyzes aldosterone synthesis. CYP11B1 encodes 11-β-hydroxylase that is involved in cortisol production. The high homology between these two enzymes is pivotal, as only aldosterone is the target in RAAS, meaning that developing a selective ASi that do not impact cortisol production is difficult (Azizi *et al.* 2013) (Figure 13).

Some preclinical studies have been led with FAD286 which was the first oral ASi agent tested in rodents. It was shown to be organ protective (Namsolleck and Unger 2014), and among other

beneficial effects to improve left ventricular structure and function in a rat model of myocardial infarction (Mulder *et al.* 2008). The first orally active ASi that has been developed and tested in humans is LCI699 by Novartis. LCI699 was safe and showed efficacy in inhibiting aldosterone. However, high dose of this compound cannot be used since it is not CYP11B2 selective, and impacts cortisol production (Calhoun *et al.* 2011).

It is suggested that aldosterone synthase inhibition may be used on top of standard of care in the context of hypertension, and might be more efficacious in protecting organs structure and functions than in decreasing blood pressure (Karns *et al.* 2013).

Important way of developing efficacious ASi is to find selective compounds for CYP11B2. It is worth noticing that targeting aldosterone synthesis involves some risks, such as accumulation of 11-deoxycorticosterone (11-DOC, precursor of aldosterone) that could act on mineralocorticoid receptor, and induce electrolyte disorders, hypotension and hypoaldosteronism (Azizi *et al.* 2013; Hargovan and Ferro 2014).

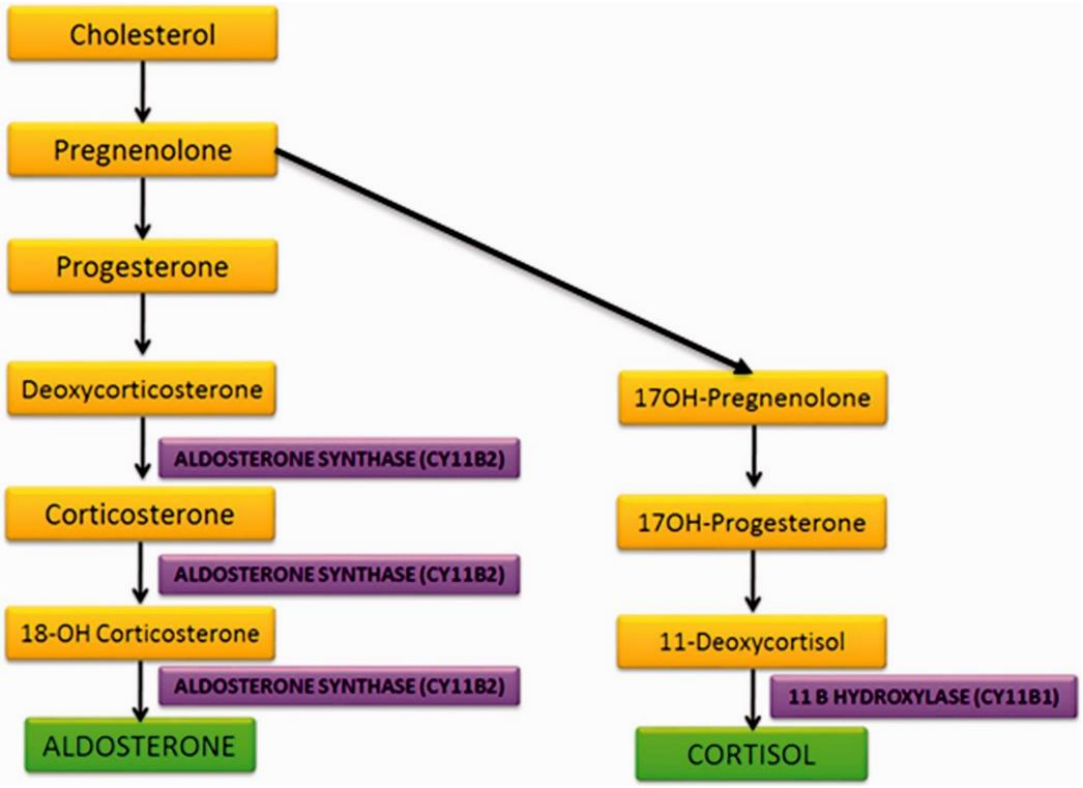

Figure 13 Schematic of adrenal steroid biosynthesis.

From (Hargovan and Ferro 2014).

1.6 MANAGEMENT OF METABOLIC SYNDROME

As metabolic syndrome is not a disease but a cluster of diseases characterized by chronic inflammation, there is no single treatment for metabolic syndrome. As a result, good clinical identification and management of patients that have metabolic syndrome are requested to adapt the treatment individually according to the advancement stage of symptoms (Wong 2005). Nevertheless, this management is difficult as no unique methodology exists to improve the whole syndrome.

Preventive measures can be efficacious for weight loss, such as physical activity, adapted diet, and as a result can have a beneficial effect on the several components of the metabolic syndrome. Still, when the preventive measures are not sufficient, generally physicians treat each component separately with drug therapies (Deen 2004). The first goal of managing patients with metabolic syndrome is to decrease their cardiovascular risk by individualizing treatment strategies according to the patient's specific risk factors (Fujioka 2006). The reason is that it is easier and faster to get reasonable improvement of the targeted disease or parameter, than making the patient only adopt a better lifestyle, which could on the long run have a beneficial effect on body weight, and as a result on the several components of the metabolic syndrome. Consequently, pharmacologic treatments are often given on top of a lifestyle modification.

There is a tool to estimate the short term cardiovascular risk in patients that have the metabolic syndrome: the Framingham algorithm. This tool classes patients in several categories based on a 10 year risk of coronary heart disease: high risk (10-year risk ≥20%), moderately high risk (10-year risk 10% to 20%), moderate risk (10-year risk 6% to 10%) and low risk (10-year risk ≤6%). This concerns patients that have no diabetes or arteriosclerotic cardiovascular disease. If so, patients belong to the high risk category (NCEP, 2002). This way of estimating the risk for patients is based on blood pressure, smoking, age, and cholesterol levels (HDL-C and total cholesterol).

Guidelines of the National Cholesterol Education Program (NCEP 2002), the eighth Joint National Commission (JNC-VIII) for blood pressure treatment (James *et al.* 2014), the National Institute of Health Obesity Initiative (NIH 1998), the American Diabetes Association (ADA) (ADA 2005) and the American Heart Association (AHA) (Grundy *et al.* 2005), should be followed by physicians for optimizing the treatments of the several components that constitute the metabolic syndrome.

In function of the Framingham score and the extent of the targeted component of metabolic syndrome, a multidisciplinary approach should be adopted for therapy as described in Table 4 (see page 70).

As stated before, lifestyle modification is the first therapy that should be preconized. Diet is a part of this therapy, and as an example, Mediterranean diet that was shown to be inversely correlated to metabolic syndrome development in the Seguimiento University of Navarra prospective cohort study (SUN) (Tortosa *et al.* 2007; Babio *et al.* 2009; Paletas *et al.* 2010). Moreover, (Lien *et al.* 2007) the Dietary Approaches to Stop Hypertension (DASH) diet plus lifestyle interventions have been shown to improve the metabolic parameters, and more particularly blood pressure, in the PREMIER study. The DASH eating plan implies many fruits and vegetables, grains, and other foods that are heart healthy and lower in sodium. Then, physical activity is central as well. As an example, physical training has been shown to reduce the skeletal muscle lipid levels and insulin resistance, regardless of BMI (Goodpaster *et al.* 2001), to decrease plasma triglycerides levels, and to reduce cardiovascular morbidity (Koplan and Dietz 1999). Regular and moderate exercise during at least 30 minutes a day is recommended by physical activity guidelines, although 60 minutes of quick walking completed by other activities is preferred (Thompson *et al.* 2003; Grundy *et al.* 2004). Daily sodium intake should also be restricted to no more than 65 to100 mmol and increased potassium intake is preconized to decrease blood pressure (Whelton *et al.* 1997; Appel *et al.* 2006). This can be reached eating many fruits and vegetable, and as a result is compatible with the DASH eating plan. A weight loss of 5 to 10% over 4 to 6 months is the expected outcome and can be targeted with the lifestyle modifications stated above. Pharmacological treatment can be given to control glycemia and improve glucose tolerance such as metformin, pioglitazone or acarbose (α-glucosidase). The aim is to reduce HbA1c below 6.5% and to reduce glycemia below 7mM in fasting conditions.

Metformin is one of the most prescribed anti-diabetic. Metformin is indicated for most of overweight and obese patients. It increases insulin sensitivity, suppresses hepatic glucose production, enhances

glucose uptake, increases fatty acid oxidation and decreases glucose absorption. Its mode(s) of action by which glycemia is decreased is still under debate (Collier *et al.* 2006; Viollet *et al.* 2012). Most of patients have reached a reduction of 1.5% in their HbA1c levels with metformin (Nathan *et al.* 2006). The most common reported adverse reaction to metformin therapy is gastrointestinal upset, including nausea, vomiting, anorexia, and diarrhea. Lactic acidosis has been reported as serious side-effect but with a low prevalence (0.01 to 0.08 case per 100 patients-year) (Bailey and Turner 1996). Moreover, this risk is even increased with the use of iodinated contrast medium (that can temporarily decease renal function) in patients, in which metformin can accumulate. This accumulation is certainly due to the inhibition of hepatic glucose production from lactate (Goergen *et al.* 2010).

Pioglitazone is an insulin sensitizer belonging to the thiazolidinedione (TZD) class. Its mode of action consists in binding to peroxisome proliferator-activated receptors (PPARs), mainly PPARγ, in cells. The drug-PPAR-complex affects the transcription of many genes. They have been shown to stimulate production of proteins like adiponectin, that increase insulin sensitivity and also to block transcription of others that enhance inflammation and insulin resistance (Chinetti *et al.* 2000; Willson *et al.* 2001; Yki-Järvinen 2004). The clinical effect of pioglitazone is to lower fasting glucose. Expected reduction of HbA1c with these medications is 0.5-1.4% (Nathan *et al.* 2006). *Via* its effect on PPARα, pioglitazone may also improve dyslipidemia by increasing HDL production (Colin *et al.* 2013).

Some rare adverse effects like hepatotoxicity have been reported, and the major limitations in prescribing TZDs to old patients, are the risk of edema, fluid retention, cardiac risk, and bone fracture (Coniff *et al.* 1995). Troglitazone, another TZD, was removed from the market in 2000 because of hepatotoxicity. Pioglitazone was also banned in 2011 from the market in France and Germany, as it has been associated with increased risks of developing bladder cancer. Moreover, rosiglitazone has been associated with an increased risk of any cardiovascular events (Hsiao *et al.* 2009).

More recently, dual PPAR (PPARα/γ also called -glitazars) showed greater efficacy in decreasing hyperglycemia and insulin resistance in type 2 diabetes especially by improving lipid metabolism and reducing diabetic microvascular complications (Keech *et al.* 2005; Chew *et al.* 2010). These compounds are still under investigation and not yet on the market. Other strategies are still under evaluation, like incretins agonism. The gastrointestinal tract produces several peptides like the incretins, glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide, that act on the pancreatic islets. GLP-1 enhances insulin secretion by β-cells and suppresses glucagon secretion by α-cells. Incretins-based therapies are essentially centered on GLP-1 analogues, and can be used as monotherapy or in combination with other anti-diabetics. Incretins induce beneficial effects not only on glycemia but also on body weight (decrease), and could be associated with other benefits like reduction of inflammation or protection of cardiovascular system (Stonehouse *et al.* 2012). Our team has been working on multi-incretin agonism strategies for many years (See annex 2).

α-glucosidase enzymes are located in the small intestine. Their function is to hydrolyze polysaccharides and oligosaccharides in monosaccharides so that they can be absorbed. Such inhibitors act in preventing this hydrolysis and decreasing the absorption of monosaccharides (Bischoff 1995). Expected reduction of HbA1c with these medications is 0.5-0.8% (Nathan *et al.* 2006). One drawback of this type of treatment is that the non-hydrolyzed carbohydrates reach the colon and are digested by bacteria and provoke diarrhea and flatulence. This important side-effect limits their utilization use, as discontinuation of the drug was seen in 24% of the cases in the STOP-Noninsulin-Dependent Diabetes Mellitus (STOP-NIDDM) (Scheppach *et al.* 1988; Chiasson *et al.* 1994; Chiasson *et al.* 2003).

Low-dose aspirin is frequently recommended to decrease the hypercoagulable state to the patients with metabolic syndrome (Shields and Hennekens 2004) , but is given based on the Framingham risk

factor category, and is excluded if the risk score is below 6%, as the benefit / risk ratio is not favorable (high hemorrhage risk as compared to expected benefit).

Regarding blood pressure control, treatment should be initiated in non-diabetic patients when systolic / diastolic blood pressure is above 140 mm Hg / 90 mm Hg, and 130 mm Hg / 80 mm Hg in diabetic patients. To reduce blood pressure, the RAAS is the first target. ACE inhibitors alone or in combination with ARBs are given as a first line therapy as it has been shown that it could be beneficial on diabetes progression and chronic kidney disease (Israili *et al.* 2007). Then the use of beta-blockers to target the sympathetic nervous system, or diuretics like thiazides can be considered as well, but have been shown to enhance the risk of diabetes development in the Antihypertensive Treatment and Lipid Profile in the North of Sweden Efficacy Evaluation (ALPINE). Nevertheless, other studies like Antihypertensive and Lipid-Lowering treatment to prevent Heart Attack Trial (ALLHAT) have shown that these compounds lower the risk of CVD events even in the patients with diabetes (ALLHAT 2002) so targeting the RAAS has been preferred (Lindholm *et al.* 2003; Ruilope *et al.* 2003; Opie and Schall 2004).

Dyslipidemia is also targeted essentially with statins, and the outcome depends on the Framingham score for the LDL-C levels, ranging from < 160 mg/dL for low-risk patients to < 100 mg/dL for high-risk patients. Vitamin B3 (niacin) or fibrates can be given on top of statins, for patients with high triglycerides levels and low HDL-C (Grundy *et al.* 2004). Vitamin B3 has been shown to be efficacious in increasing HDL-C levels and HDL size (Kashyap *et al.* 2002), and reducing triglycerides and LDL-C levels (NCEP, 2002; Gouni-Berthold and Berthold 2013)

Finally, bariatric surgery can be preconized if body mass index (BMI) is superior to 40 or up to 35 with minimum one of the features cited above, such as T2D and for patients for which conventional treatment were not efficacious enough to lose weight (Wilson and Grundy 2003).

Table 4 **Multidisciplinary approach to the metabolic syndrome.**

From (Kaur 2014) – ACEIs: angiotensin converting enzyme inhibitors; ARBs: aldosterone receptor blockers; BP: blood pressure; CVD: cardiovascular disease; CRP: C reactive protein; HDL-C: high density lipoprotein – cholesterol; LDL-C: low density lipoprotein – cholesterol; MetS: metabolic syndrome; T2DM: type 2 diabetes mellitus.

A: assessment	Calculate Framingham risk score: *high risk* (10-year risk ≥20%), *moderately high risk* (10-year risk 10% to 20%), or *lower to moderate risk* (10-year risk ≤10%). Make diagnosis of MetS using the diagnostic criteria.
A: aspirin	High risk: aspirin definitely beneficial. High-intermediate risk (10–20%): aspirin likely to be beneficial. Low-intermediate risk (6–10%): "individual clinical judgment", depending on sex and risk of bleeding. Low risk (<6%): Risk of haemorrhage outweighs the benefits.
B: BP control	Initiate treatment: categorical hypertension (BP ≥ 140/≥90 mm Hg). Patients with established diabetes (≥130/≥80 mm Hg). ACEIs/ARBs first line agent may reduce incident diabetes mellitus. Beta-blockers and thiazides may have an adverse effect on impaired glucose tolerance but outweighed by the benefits of reaching BP goal and lowering the risk of CVD events.
C: cholesterol First target: LDL Second target: non-HDL Third target: HDL Fourth target: CRP	Statin to achieve LDL-C <100 mg/dL in high-risk, <130 mg/dL in intermediate-risk, and <160 mg/dL in low risk patients. Statin intensification, consider niacin and/or fibrates once statin maximized. Consider fibrates, especially for those with combined hypertriglyceridemia/low HDL-C. Consider further reduction in LDL-C with statin therapy to mitigate a risk of low HDL-C, consider niacin. Statin therapy for those with high sensitivity CRP (hsCRP) ≥3 mg/dL.
D: diabetes prevention/diet	Intensive lifestyle modification is the most important therapy. Weight reduction of 5–10% of preintervention weight over a period of four to six months. Sodium intake of <65–100 mmol/day with a goal of 90–120 mmol of potassium per day. Mediterranean diet: high consumption of fruits, vegetables, legumes, and grains, moderate alcohol intake, a moderate-to-low consumption of dairy products and meats/meat products, and a high monounsaturated- to-saturated fat ratio. DASH diet: rich in fruits, vegetables, and low-fat dairy products, and low in saturated and total fat intake. Consider low glycemic index food, complex unrefined carbohydrates, viscous soluble fibres, protein intake of 10–35% of total calorie intake and 25% to 35% of calories as total fat. Metformin is second line in delaying the onset of T2DM. Thiazolidinediones (Pioglitazones) and alpha-glucosidase inhibitors (Acarbose) have shown benefit in smaller studies and are therefore third line.
E: exercise	Daily moderate intensity activity of minimum 30 minutes for most days of the week. Recommend use of pedometer with goal >10,000 steps/day.

1.7 NEW THERAPEUTIC APPROACHES

Although classical strategies targeting RAAS (ACEi, ARBs) have shown efficacy in reducing diabetic nephropathy (Santoro *et al.* 1992; Brenner *et al.* 2001; Appel *et al.* 2003; Terra 2003; Roscioni *et al.* 2014), their benefits of combination therapy in chronic kidney disease patients remains controversial, as already mentioned above in the Section 1.5.4 (Fernandez Juarez *et al.* 2013; Fried *et al.* 2013). As the benefit / risk ratio for combination between ACEi and ARB is not always favorable (especially in the kidney disease patients), but as the idea to target several components of the RAAS remains valuable, which is displayed by the combination between eplerenone and another class, it is necessary to find other innovative treatments targeting the RAAS. One approach is the development of selective aldosterone synthase inhibitor, as it is suggested that ASi may be used on top of standard of care in the context of hypertension, and might be more efficacious in protecting organs structure and functions than in decreasing blood pressure (Karns *et al.* 2013). Moreover, a selective aldosterone synthase inhibitor could show better results in terms of targeting the RAAS as it is expected to blunt aldosterone production, avoiding its genomic and non-genomic effects, whereas the MRA cannot avoid the aldosterone effects not mediated by MR if the hypothesis of an existing membrane-associated aldosterone receptor is verified (non-genomic effects of aldosterone not dependent of the mineralocorticoid receptor).

Aside from the RAAS, another approach is to use anti-inflammatory and antioxidant compounds, since chronic inflammation and production of ROS are central in the development of the triad metabolic syndrome, chronic kidney disease and cardiovascular disease. Bardoxolone methyl is an activator of Nrf2 and Keap-1 pathway that has been shown to activate the cellular antioxidant process (Jaramillo and Zhang 2013; Zoja *et al.* 2013). Bardoxolone has been evaluated in animal models of chronic kidney disease and clinical studies in chronic kidney disease patients. In preclinical studies, bardoxolone or bardoxolone analogues have shown improvement of structure and kidney function in acute kidney injury mice models (Aleksunes *et al.* 2010; Wu *et al.* 2011) as well as in

chronic studies involving 5/6 nephrectomy (Heloisa *et al.* 2005) or Zucker Diabetic Fatty rats (ZDF) (Chin *et al.* 2013).

From a clinical point of view, bardoxolone has shown efficacy in improving kidney function during short term studies in patients with chronic kidney disease and type 2 diabetes (Pergola *et al.* 2011; Pergola *et al.* 2011) but not in end stage renal disease patients.

It has moreover been associated with increased incidence of cardiovascular death in patients with stage 4 chronic kidney disease, in the Bardoxolone methyl EvAluation in patients with Chronic kidney disease and type 2 diabetes mellitus: the Occurrence of renal eveNts (BEACON) study, leading to stop the study earlier (de Zeeuw *et al.* 2013; Lambers Heerspink *et al.* 2013). This remains controversial as it has recently been described for patients with no heart failure hint prior to hospitalization, that the risk for heart failure was the same in patients treated with bardoxolone than in patients receiving placebo (Chin *et al.* 2014).

Although these controversial effects have led to a reassessment of the therapeutic potential of bardoxolone in chronic kidney disease as well as other indications (de Zeeuw *et al.* 2013; Rossing 2013; Zoja *et al.* 2013), this class of drugs could be further improved to enhance organ protection, on top of the classical therapeutics.

2. EXPERIMENTAL WORK : EVALUATION OF THE CARDIAC AND RENAL FUNCTIONS OF THE MALE ZSF1 RAT

Ce second chapitre porte sur l'aspect expérimental de nos travaux. Avant de pouvoir tester l'effet protecteur de composés sur les fonctions rénale et cardiaque, nous devions évaluer l'évolution de ces fonctions avec l'âge chez le rat ZSF1. La fonction cardiaque notamment n'avait été que peu décrite dans la littérature, c'est pourquoi nous l'avons évaluée *via* échocardiographie. Cette technique ultrasonore est utilisée chez l'Homme, et présente de nombreux avantages par rapport à d'autres techniques d'imagerie. En particulier, elle permet de mesurer la fonction cardiaque de manière non-invasive et en un temps court. Le principe de fonctionnement de l'échographie est succinctement décrit dans ce chapitre, ainsi que les différents modes utilisés lors de nos études pour évaluer la fonction cardiaque (B-mode, M-mode, et Doppler). Après un éditorial (Benardeau *et al*. 2013) (*cf*. page 76) sur les modèles animaux de syndrome métabolique, la première étude de ce chapitre a donc consisté à évaluer la fonction cardiaque avec l'âge chez le rat ZSF1, et a donné lieu à une première publication (Riboulet *et al.* 2013) (*cf*. page 93). Il est apparu que dans ce modèle, la fonction systolique était préservée alors que la fonction diastolique était dégradée assez tôt. Nous décrivons ensuite ce qui a été observé pour la fonction rénale dans ce modèle dont la dégradation ne survient que tardivement, vers un an, avec l'apparition d'une sclérose glomérulaire marquée. De ce fait, sachant que la fonction cardiaque était assez peu impactée lors du vieillissement chez le rat ZSF1, et que la fonction rénale n'était touchée que tardivement, nous avons mis en place deux stratégies pour accélérer et accentuer le degré de sévérité de ces défaillances.

La première méthode utilisée a été la néphrectomie unilatérale (UNx), et nous a permis de mettre en évidence une exacerbation de la dysfonction rénale chez le rat ZSF1, mais pas de la dysfonction cardiaque. Ce travail a donné lieu à la rédaction d'un second manuscrit (short communication, actuellement soumise à *Journal of the American Society of Nephrology*) (*cf*. page 129). Puis, nous avons testé l'efficacité de l'antioxydant bardoxolone sur la protection rénale chez le rat ZSF UNx, et montré qu'il était aussi efficace que le composé de référence lisinopril, un inhibiteur de l'enzyme de conversion de l'angiotensine I. Cette étude a donné lieu à un 3° manuscrit (à soumettre à *Pflügers archiv*) (*cf*. page 153) dans lequel nous émettons également l'hypothèse, au vu des résultats obtenus,

que l'effet protecteur du bardoxolone sur la fonction rénale était partiellement dû à la perte de poids engendrée par la réduction de la prise alimentaire chez le rat ZSF1.

La seconde méthode a consisté en l'infusion d'AngII afin de produire un stress hémodynamique, et un stress hormonal par la synthèse massive d'aldostérone. Ce modèle que nous appelons ZSF-AngII a montré une fonction rénale, ainsi qu'une fonction cardiaque, rapidement altérées. Par conséquent, ce modèle ZSF-AngII nous a permis de tester l'effet de l'antagoniste des récepteurs aux minéralocorticoïdes éplérénone ainsi que d'un inhibiteur de l'aldostérone synthase innovant sur la protection rénale mais aussi cardiaque, et de montrer une meilleure protection de la part de l'inhibiteur de l'aldostérone synthase. Cette étude a donné lieu à un 4° manuscrit (à soumettre à *Kidney International*) (*cf*. page 205).

2.1 ANIMAL MODELS OF METABOLIC SYNDROME

In order to test efficacy of new compounds in the field of metabolic syndrome, type 2 diabetes, cardiovascular disease and chronic kidney disease, the first need was to find a translational animal model, *i.e.* which mimics the human pathophysiological mechanisms leading to kidney and cardiovascular damages, in a context of metabolic syndrome. On that purpose, we published an editorial in the Journal of Experimental and Applied Animal Science in 2013 to review the main animals models of metabolic syndrome and type 2 diabetes used in the research area.

Editorial (*Journal of Experimental and Applied Animal Science*, 2013, 1(1):152-155)

Animal models of metabolic syndrome and associated co-morbidities

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Type 2 Diabetes (T2D) is recognized by the World Health Organization as a global epidemic affecting 284-347 million people in the world depending on the inclusion criteria used (Danaei et al. 2011; American Diabetes Association 2013). Co-morbidities associated with T2D are hypertension, cardiovascular diseases (CVD) (Castelli 1984; Wilson et al. 2005) and diabetic nephropathy (DN) (Shah et al. 2009; Shao et al. 2013). There is an unmet need for therapies curing or preventing the progression of T2D and combined pathologies (Grundy 2008).

In the metabolic diseases field, transgenic or chemically-induced models (mainly rodents) were characterized and focused on one disease or disturbance (i.e. dyslipidemia, T2D, obesity) or pathway abnormalities (insulin resistance, glucose intolerance). The main limitation for the use of those models is the lack of translatability to human T2D and co-morbidities. Metabolic syndrome (MS, MetS, Syndrome X) was described years ago as a combination of at least 3 diseases or affections out of abdominal obesity, dyslipidemia, high fasting blood glucose, glucose intolerance, high blood pressure, high cholesterol (Reaven 1993; Hanson et al. 2002; Grundy et al. 2004) and re-defined more recently (Alberti et al. 2007). The need for new predictive animal models to characterize compounds that efficiently prevent or resume the MS and co-morbidities is more evident than ever. Public-private partnerships in diabetes research are taking on this challenge: Identifying and developing predictive animal models of MS, T2D and its complications and defining surrogate markers (biomarkers, genetic markers, imaging markers) as tools for disease progression and for the evaluation of compound efficacy, are key objectives of the SUMMIT consortium* within the Innovative Medicines Initiative (IMI). The closest animal model to human MS ever characterized is the non-human primate Rhesus Monkey. This model is characterized by obesity, T2D, insulin resistance, hypertension and dyslipidemia that progressively develop with age (Cusumano et al. 2002; Tigno et al. 2004) and is sensitive to Standards of Care (Hansen 2012; Brautbar and Ballantyne 2011; Hansen et al. 2011). So far, no equivalent <u>but</u> smaller animal model (i.e. rodents) of MS and co-morbidities exists. The summary of rodent models of metabolic disturbances below does not pretend listing all models, but

reflects the most widely used. For a more extensive review the reader is directed to the work published by Sima and Shafir in their book “chronic complications in diabetes” (Sima 2000).

Still today, the most commonly used models of T2D are the genetically male Zucker Diabetic (ZDF) rats and male or female db/db mice (both characterized by leptin disorders). Male ZDF rats develop features of MS and T2D (glucose intolerance, insulin resistance, hyperinsulinemia, β-cells dysfunction and hyperlipidemia associated with obesity) (Tofovic and Jackson 2003). Even though this model rapidly develops overt T2D, it does not develop hypertension (Prabhakar et al. 2007) and it only develops mild nephropathy without mimicking features of human DN (Marsh et al. 2007; Pollock and Carmines 2008). Similar to the ZDF rats, db/db mice exhibit features of T2D but do not develop severe kidney deterioration (Tesch and Lim 2011).

Another extensively used model of T2D since the 70’ is the Streptozotocin (STZ)-induced diabetes (De Nicola et al. 1976; Trancrède et al. 1983). T2D is induced by either single or repeated injections of STZ (under specific feeding or fasted state) that lead to partial or sub-total pancreatic β-cells destruction. Despite clear hyperglycemia, STZ-treated animals are not prone to develop hypertension, cardiac and renal dysfunction (Bilan et al. 2011).

eNOs_db/db and ^{Ins2}Akita mice are models characterized by T2D, accelerated DN and retinal complications (Zhao et al. 2006; Barber et al. 2005). Goto-Kakizaki rat, a non-obese T2D model, mimics MODY diabetes (Movassat et al. 1997) although without features of DN. Finally, models of hypertension (Spontaneously Hypertensive Heart Failure, Spontaneously Hypertensive Rat and Dahl-Salt rats) failed to exhibit hyperglycemia or T2D and/or DN (Koletsky 1973; Michaelis et al. 1986; Heyen et al. 2002). Overall, the existing rodent models of hyperglycemia or T2D rarely develop the main co-morbidities: CVD and DN.

A relatively recent model has given some hope in the MS syndrome field: the male ZSF1 (Zucker fatty/Spontaneously hypertensive heart failure F1 hybrid) rat is characterized by insulin resistance, mild hypertension, obesity and DN, i.e. all features that are common complications of T2D in humans (Tofovic and Jackson 2003; Griffin et al. 2007; Joshi et al. 2009; Mohanan et al. 2011). Promising

results with respect to CVD demonstrated that this model develops left ventricular diastolic dysfunction (Russo et al. 2010). Our recent work (Riboulet et al. 2013) however shows that the ZSF1 rats neither develop major nor advanced cardiac dysfunction. It is believed that exacerbation of MS of ZSF1 rats could lead to T2D, CVD and DN (i.e. cardio-renal syndrome) and diabetic retinopathy and would constitute a very good translational model to reflect the human MS.

* In SUMMIT 6 pharma partners and 19 academic institutions and 1 SME are working towards developing innovative approaches to make clinical trial testing of novel medications in diabetic vascular complications faster and more efficient. SUMMIT receives support from the Innovative Medicines Initiative Joint Undertaking, resources of which are composed of financial contribution from the European Union's Seventh Framework Program (FP7/2007-2013) and EFPIA companies' in kind contribution.
For further details – please visit: www.imi-summit.eu and www.imi.europa.eu.

References

Alberti, G, Zimmet P, Kaufman F, Tajima N, Silink M, Arslanian S, Womg G, Bennett P, Shaw J, Caprio S (2007). METABOLIC SYNDROME - in children and adolescents. IDF communication.

American Diabetes Association (2013). Diagnosis and Classification of Diabetes Mellitus. Diabetes care 36(Supplement 1): S67-S74.

Barber AJ, Antonetti DA, Kern TS, Reiter CEN, Soans RS, Krady JK, Levison SW, Gardner TW, Bronson SK (2005). The Ins2Akita Mouse as a Model of Early Retinal Complications in Diabetes. Investigative Ophthalmology & Visual Science 46(6): 2210-2218.

Bilan VP, Salah EM, Bastacky S, Jones H B, Mayers RM, Zinker B, Poucher SM, Tofovic SP (2011). Diabetic nephropathy and long-term treatment effects of rosiglitazone and enalapril in obese ZSF1 rats. The Journal of endocrinology 210(3): 293-308.

Brautbar A and Ballantyne CM (2011). Pharmacological strategies for lowering LDL cholesterol: statins and beyond. Nat Rev Cardiol 8(5): 253-265.

Castelli WP (1984). Epidemiology of coronary heart disease: The Framingham study. The American journal of medicine 76(2, Part A): 4-12.

Cusumano AM, Bodkinm NL, Hansen BC, Iotti R, Owens J, Klotman PE, Kopp JB (2002). Glomerular hypertrophy is associated with hyperinsulinemia and precedes overt diabetes in aging rhesus monkeys. American journal of kidney diseases 40(5): 1075-1085.

Danaei G. Finucane MM, Lu Y, Singh GM, Cowan MJ, Paciorek CJ, Lin JK, Farzadfar F, Khang Y-H, Stevens GA, Rao M, Ali MK, Riley LM, Robinson CA, Ezzati M (2011). National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants. The Lancet 378(9785): 31-40.

De Nicola AF, Del Castillo FO, Foglia EJ (1976). The Influence of Streptozotocin Diabetes on Adrenal Function in Male Rats. Horm Metab Res 8(5): 388-392.

Griffin K A, Abu-Naser M, et al. (2007). Dynamic blood pressure load and nephropathy in the ZSF1 (fa/facp) model of type 2 diabetes. American Journal of Physiology - Renal Physiology 293(5): F1605-F1613.

Grundy SM, Brewer HB Jr, Cleeman JI, Smith SC Jr, Lenfant C (2004). Definition of metabolic syndrome: Report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. Circulation 109(3): 433-438.

Grundy SM. (2008). Metabolic Syndrome Pandemic. Arteriosclerosis, thrombosis, and vascular biology 28(4): 629-636.

Hansen BC (2012). Investigation and Treatment of Type 2 Diabetes in Nonhuman Primates. Animal Models in Diabetes Research Humana Press. 933: 177-185.

Hansen B, Tigno X, Benardeau A, Meyer M, Sebokova E, Mizrahi J (2011). Effects of aleglitazar, a balanced dual peroxisome proliferator-activated receptor alpha/gamma agonist on glycemic and lipid parameters in a primate model of the metabolic syndrome. Cardiovascular Diabetology 10(1): 7.

Hanson RL Imperatore G, Bennett PH, Knowler WC (2002). Components of the metabolic syndrome and incidence of type 2 diabetes. Diabetes, 51(10): 3120-3127.

Heyen JRR, Blasi ER, Nikula K, Rocha R, Daust HA, Frierdich G, Van Vleet JF, De Ciechi P, McMahon EG
Rudolph AE (2002). Structural, functional, and molecular characterization of the SHHF model of heart failure. American Journal of Physiology - Heart and Circulatory Physiology 283(5): H1775-H1784.

Joshi D, Gupta R, Dubey A, Shiwalkar A, Pathak P, Gupta RC; Chauthaiwale V, Dutt C (2009). TRC4186, a Novel AGE-breaker, improves Diabetic Cardiomyopathy and Nephropathy in Ob-ZSF1 Model of Type 2 Diabetes. Journal of Cardiovascular Pharmacology 54(1): 72-81.

Koletsky S (1973). Obese spontaneously hypertensive rats—A model for study of atherosclerosis. Experimental and Molecular Pathology 19(1): 53-60.

Marsh SA, Powell PC, Agarwal A, Dell'Italia LJ, Chatham JC (2007). Cardiovascular dysfunction in Zucker obese and Zucker diabetic fatty rats: role of hydronephrosis. American Journal of Physiology - Heart and Circulatory Physiology 293(1): H292-H298.

Michaelis OE 4th, PD, Hansen CT, Canary JJ, Werner RM, Carswell N (1986). Insulin-independent diabetes mellitus (type II). Spontaneous hypertensive/NIH-corpulent rat." Am J Pathol. 123(2): 398-400.

Mohanan A, Gupta R, Dubey A, Jagtap V, Mandhare A, Gupta RC, Chauthaiwale V, Dutt C (2011). TRC120038, a Novel Dual AT1/ETA Receptor Blocker for Control of Hypertension, Diabetic Nephropathy, and Cardiomyopathy in ob-ZSF1 Rats. International Journal of Hypertension12: Article ID 751513

Movassat J, Saulnier C, Serradas P, Portha, B. (1997). Impaired development of pancreatic beta-cell mass is a primary event during the progression to diabetes in the GK rat. Diabetologia 40(8): 916-925.

Pollock JS and Carmines PK (2008). Diabetic nephropathy: nitric oxide and renal medullary hypoxia. American Journal of Physiology - Renal Physiology 294(1): F28-F29.

Prabhakar S, Starnes J, Shi S, Lonis B, Tran R (2007). Diabetic Nephropathy Is Associated with Oxidative Stress and Decreased Renal Nitric Oxide Production. Journal of the American Society of Nephrology 18(11): 2945-2952.

Reaven GM (1993). Role of insulin resistance in human disease syndrome X: an expanded definition. Annual review of medicine, 44: 121-131.

Riboulet W, Hainaut E, Verry P, Lambotte J, Jacobsen B, Lartaud I, Bénardeau A, Conde-Knape K (2013). Longitudinal assessment of heart function of ZSF1 rats by echocardiography. Journal of Experimental and Applied Animal Science 1(1): 125-139.

Russo C, Jin Z, Homma S, Rundek T, Elkind MSV, Sacco RL, Di Tullio, Marco R (2010). Effect of diabetes and hypertension on left ventricular diastolic function in a high-risk population without evidence of heart disease. European journal of heart failure 12(5): 454-461.

Shah IM, Mckay SP, McKay GA (2009). Therapeutic Strategies in the Treatment of Diabetic Nephropathy - a Translational Medicine Approach. Current Medicinal Chemistry 16(8): 997-1016.

Shao N, Kuang HY, Wang N, Gao XY, Hao M, Zou W, Yin HQ (2013). Relationship between Oxidant/Antioxidant Markers and Severity of Microalbuminuria in the Early Stage of Nephropathy in Type 2 Diabetic Patients. Journal of Diabetes Research 2013: 6.

Sima, Ed. (2000). Chronic complications in diabetes. Animal models and chronic complications. Detroit, harwood academic publishers

Tancrède G, Rouseau-Migneron S, Nadeau A (1983). Long-term changes in the diabetic state induced by different doses of streptozotocin in rats. British Journal of Experimental Pathology 64(2): 117-123.

Tesch GH and Lim AK (2011). Recent insights into diabetic renal injury from the db/db mouse model of type 2 diabetic nephropathy. American journal of physiology. Renal physiology 300(2): F301-310.

Tigno XT, Gerzanich G, Hansen BC. (2004). Age-Related Changes in Metabolic Parameters of Nonhuman Primates. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences 59(11): 1081-1088.

Tofovic SP and Jackson EK (2003). Rat models of the metabolic syndrome. Methods in molecular medicine 86: 29-46.

Wilson PWF, D'Agostino RB Parise H, Sullivan L, Meigs JB (2005). Metabolic Syndrome as a Precursor of Cardiovascular Disease and Type 2 Diabetes Mellitus. Circulation 112(20): 3066-3072.

Zhao YF, Feng D, Chen C. (2006). Contribution of adipocyte-derived factors to beta-cell dysfunction in diabetes. The International Journal of Biochemistry & Cell Biology Diabetes: New Research and Novel Therapies 38(5-6): 804-819.

Among the different metabolic syndrome rodent models available so far, obese Zucker fatty/Spontaneously hypertensive heart failure F1 hybrid (ZSF1) rat was highlighted as a model developing metabolic syndrome and some features of kidney disease (Tofovic SP 2000; Tofovic and Jackson 2003). ZSF1 rat may be the best available rodent model mimicking human diabetic nephropathy. Indeed, this rat model meets currently accepted criteria for progressive experimental diabetic renal disease in rodents (Bilan *et al.* 2011).

2.2 USEFULNESS OF THE ZSF1 MODEL AND AIM OF THE EXPERIMENTAL WORK

Although the ZSF1 rat model was assumed to be the best available for metabolic syndrome, the evaluation of cardiac (dys) function needed to be investigated. This aspect deserved to be explored in order to estimate whether this model could be considered as a cardio-renal model. Moreover, such a model could be useable if its pathological alteration would develop in a reasonable time frame for pharmaceutical industry, which was not the case as published in the first reports (Tofovic SP 2000; Tofovic and Jackson 2003). Indeed, projects cannot afford to have models that can be only used at one year of age, knowing that the animals can only be delivered at 13 weeks of age at the latest by the provider. This is not compatible with the flexibility and the number of experiments required in the pharmaceutical industry. The possible limitation of such “built” model could be that accelerating the disease might not fully mimic the human reality. Still, as it is a model, it has to be consistent with the industry research requirements, and we ensure that the translational aspect between animal and human is still meaningful.

Consequently, the aims of this work were:

1/ to evaluate the age-dependent changes in physiopathology of ZSF1 rats. More specifically, we evaluated heart function by echocardiography, in a longitudinal manner.

2/ to exacerbate both cardiac and renal impairments of ZSF1 rats in order to increase the extent of the cardio-renal pathology and to shorten its development. This achievement was key to assess the efficacy of new compounds on organ protection (kidney, heart) in the context of metabolic syndrome. To do so, two options were explored to exacerbate and accelerate the deterioration of cardiac and renal functions, first surgically, then chemically.

On one hand, we explored the impact of unilateral nephrectomy (UNx), a surgical method to induce renal failure in ZSF1 rats. The UNx-ZSF model was characterized longitudinally for its cardiac and renal functions. Effects of the new kidney protective drug, bardoxolone, were tested in this UNx model, head to head with standard of care Lisinopril (ACEi).

On the other hand, subcutaneous and continuous administration of AngII to ZSF1 rats was performed. This hormone was used to enhance both cardiac and renal impairments, *via* hemodynamic and hormonal stress. Finally, the efficacy of eplerenone and an in-house CYP11B2 selective ASi were tested in this ZSF1-AngII-infused model.

Before testing new molecules' effects on cardiovascular and renal functions, we needed to evaluate how the cardiac function evolved along aging in the selected animal model (ZSF1). Actually, these features were only briefly described and incompletely characterized. For that purpose we used non-invasive methodology similar to that used in humans: the echocardiography.

2.3 ZSF1 RAT BACKGROUND

The ZSF1 rat model originated from crossing lean female ZDF (ZDF +/fa) with lean male SHHF (Spontaneously Hypertensive Heart Failure) (SHHF/Mcc, +/facp), *cf. infra*.

The ZDF rat develops features of metabolic syndrome, as found in human, like obesity, glucose intolerance, hyperglycemia, hyperinsulinemia, β-cells dysfunction and hyperlipidemia (Tofovic and Jackson 2003). However, this model does not develop hypertension with aging (Prabhakar *et al.* 2007). Furthermore, ZDF rats develop mild nephropathy with hydronephrosis (Marsh *et al.* 2007) which is not representative of the human diabetic population wherein glomerular and mesangial expansion, lesions of the tubules, and decrease in the filtration rate are found (Remuzzi *et al.* 2006; Pollock and Carmines 2008).

The SHHF rat model develops hypertension leading to congestive heart failure (Heyen *et al.* 2002) although with a much milder metabolic syndrome than the recently developed hybrid ZSF1 rat model (Tofovic and Jackson 2003).

The ZSF1 rat model is characterized by 2 mutations on the leptin receptor fa discovered by Zucker in 1961, and cp described by Koletsky, which leads to leptin resistance (and not leptin deficiency) responsible for obesity as in humans (Schwartz *et al.* 2000). Male ZSF1 model combines insulin resistance, mild hypertension, diabetic nephropathy and obesity, all features that are common in type 2 diabetes and metabolic syndrome complications (Tofovic and Jackson 2003; Joshi *et al.* 2009). However it has also been reported that hypertension and type 2 diabetes are associated with impaired left ventricular diastolic function, independent of body weight (Russo *et al.* 2010). Till now,

most of the published studies on the ZSF1 model mainly explored characteristics of metabolic changes (Tofovic and Jackson 2003) and to a certain extent on renal structure and function (Tofovic SP 2000). None of them evaluated the longitudinal evolution of the cardiac function by echography in this animal model.

2.4 ECHOCARDIOGRAPHY

Echography is a tool based on ultrasounds, *i.e.* sound waves that are not detectable by the human ear with frequencies higher than 20 KHz. Sound is a mechanical wave created by a vibrating object, is propagated through a medium. This is as well a pressure wave, as it propagation induces repeated patterns of high and low pressure regions induced by the motion of particles in a direction parallel to the source beam. Ultrasonography for diagnosis usually uses transducers that emit, with frequencies between 1 and 20 MHz, and also receive the waves coming back from the organs. The penetration of the ultrasounds decreases while the spatial resolution gets better with the increase in the frequency, (Cootney 2001). This explains why in rodents, the probe frequencies are higher than the ones used is Human, as the required examination depth is lower, but a better spatial resolution is needed (smaller structures than in Human).

At these high sound frequencies, sound waves are produced by the probe by electric pulse, and transmitted through soft tissue. In this methodology, the tissue acoustic impedance is the principle used to produce images. Acoustic impedance of a tissue is the product of the tissue density and the transmission velocity of sound. This transmission velocity of sound has been shown to be almost uniform around 1540 $m.^{s-1}$ in blood and tissues (Merritt 1998), meaning that the tissue density is determining the impedance of a tissue. This imaging modality is based on the different tissues densities: sound waves are reflected when two tissues with different densities are located next to each other, as an acoustic impedance mismatch is created (Cootney 2001). For example, bones and

soft tissue interfaces are highly reflective, as their densities are very different, *i.e.* with a large mismatch in their acoustic impedance. This explains the echogenicity of tissues: bone tissue has very high acoustic impedance as compared to soft tissue. Thus, when the ultrasound beam intersects a bone tissue, the ultrasound wave is reflected back to the transducer giving a very bright image, preventing imaging of deeper structures. One transmitted pulse gives rise to a train of received echoes. By timing how long they take to get back to the probe and according to their amplitude, an image can be constituted in shades of grey (B-Mode, see below).

Temporal resolution is defined as the ability to distinguish two events in time, and is of importance for examination of organs that are in motion, like heart. This parameter can be set by the operator and consists in the number of images acquired per second (in Hz). In rodents, and especially in mice, heart rates can go up to 600 beats per minute, meaning that evaluating cardiac function in mice requires the possibility to set the ultrasound scan with high temporal resolution, to get as many images of the cardiac cycle as possible. On the contrary, imaging kidney which is a relatively static organ does not require such high temporal resolution (Cootney 2001).

Bi-dimensional echocardiography (B-Mode, Brightness mode) is the basic mode of two-dimensional imaging, in shades of grey produced according to the anatomical site of examination that is moving in real time. B-mode has been used to determine organ size and appearance and can be used for in motion organ function determination if the temporal resolution is sufficient.

Motion mode (M-Mode) is used for the analysis of moving organs, and is commonly used in echocardiography. M-mode imaging is obtained with a single ultrasound beam transmitted through the heart, and the resulting image is displayed over time. M-mode coupled to B-mode allows visualizing -in real-time- cardiac structures in motion (Figure 14). This is also a method to measure the heart dimensions in order to assess systolic function, *i.e.* ejection fraction in our case, in a parasternal short axis view (see Figure 1 of publication 1: LONGITUDINAL ASSESSMENT OF HEART FUNCTION OF ZSF1 RATS BY ECHOCARDIOGRAPHY).

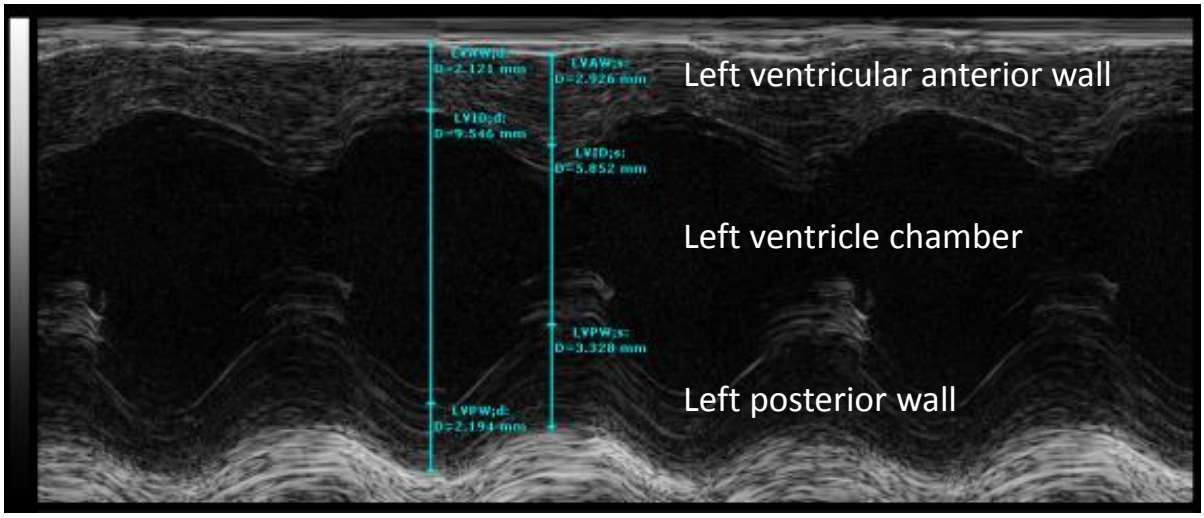

Figure 14 Left ventricular M-mode spectrum of 32 weeks-old male ZSF1 rat obtained in a parasternal short axis view.

Spectral Doppler and color flow Doppler imaging modes are based on the Doppler Effect principle: when a moving object reflects the sound wave, the frequency of this reflected sound wave changes, and this shift represents the Doppler Effect. If the object is moving away from the transducer, the frequency will be inferior to the one of the initial signal, and if the moving object comes towards the transducer, the frequency of the reflecting signal will be increased. This is true for blood cells, and as a result, this Doppler mode allows determining blood flow velocity and direction. The difference of frequencies is called the Doppler shift (Merritt 1998), and the greater the Doppler shift, the greater the velocity of the flow blood. To avoid Doppler shift attenuation, the ultrasound beam and the flowing blood have to be aligned and parallel, and this alignment has to be tuned by the operator (Cootney 2001). Practically, the B-mode is used to find the vessel in which the flow will be measured, the Doppler mode is triggered and the angle is tuned by the operator. On the screen, a velocity chart is displayed over time and allows measuring several parameters. In our case, the E (early passive filling of the left ventricle) and A (efflux due to atrial contraction) waves were measured in the left ventricle in a four chambers view (see Figure 1 of publication 1: LONGITUDINAL ASSESSMENT OF HEART FUNCTION OF ZSF1 RATS BY ECHOCARDIOGRAPHY), which allows calculation of the E/A ratio

(Figure 15). E/A ratio reflects the diastolic function (see Figure 5 of publication 1: LONGITUDINAL ASSESSMENT OF HEART FUNCTION OF ZSF1 RATS BY ECHOCARDIOGRAPHY).

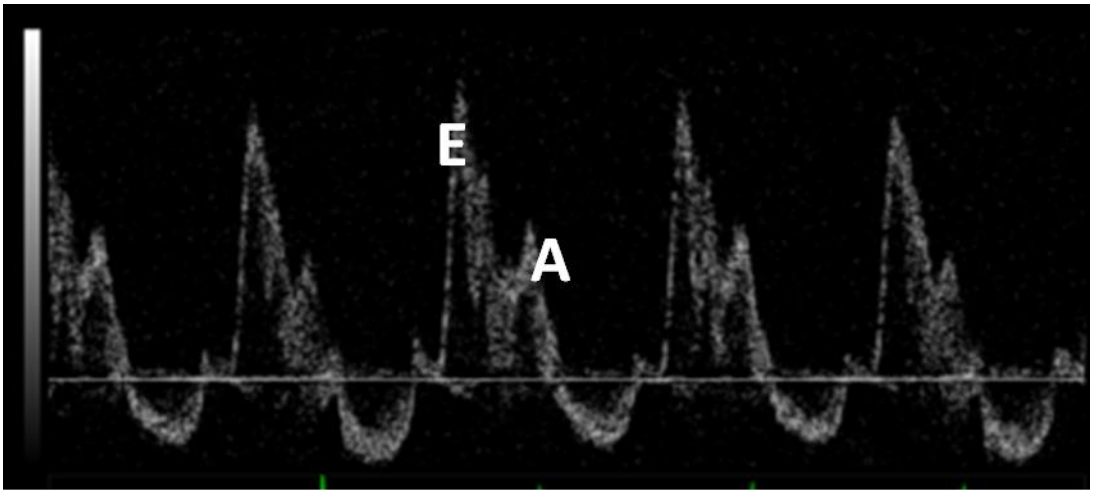

Figure 15 Left ventricular Doppler mode spectrum of 32 weeks-old male ZSF1 rat obtained in a four-chambers view. E and A waves represent respectively the early and late filling phases of the left ventricle.

This imaging modality has been increasingly used over the past years, since the exam is quick and is free of harmful radiation, in real time, affordable. It can thus evaluate blood flow, in contrast to other imaging modalities such as magnetic resonance imaging (Pearlman *et al.* 2007). Echocardiography is a useful non-invasive tool used to longitudinally evaluate changes in cardiac function in the same cohort of animals. The same non-invasive approach is used in humans and allows depicting cardiac function changes in real time and in a quick manner (Scherrer-Crosbie and Thibault 2008).

According to the recommendations of the Journal of the American Society of Echocardiography, one of the parameters to be measured in clinical trials to assess systolic function is ejection fraction, while the E/A ratio is a good indicator for diastolic function in human beings (Gottdiener *et al.* 2004). In humans, it is known that ejection fraction can be kept unchanged in spite of diastolic dysfunction

(Fiorini *et al.* 1995). These parameters are also recommended to assess both systolic and diastolic functions in preclinical studies (Hanton *et al.* 2008).

Some disadvantages exist with echocardiography. For example, in obese patients / animals, fat is very echogenic, meaning that the ultrasounds are easily attenuated through the fat layer, which renders good quality images difficult to get. This is also true for air or gas, as “shadowing” effects can be a consequence. Then, the quality of the images and clips are also operator dependent. Operator has to be well-trained, to allow reproducible assessments. Finally, resolution is increasing but is still below that of magnetic resonance imaging.

Despite those limitations, we chose this technique to evaluate cardiac function as we wished to check the longitudinal evolution, *i.e.* at different ages in the same animal. Moreover, this is a translational methodology (used in humans) and we thought it was a suitable way to follow the 3Rs (refine, reduce, replace). Additionally to cardiac function, we examined the impact of aging and progression of metabolic syndrome disease on detailed structural changes of other organs of ZSF1 rats.

The echocardiography is a continuously **refined** method as it is not painful or invasive, used in humans as well, meaning that a lot of innovation is dedicated for that purpose. Then we wanted to use the methodology which **reduces** at best the number of animals. As we can perform several assessments on the same animal along aging (not invasive as compared to pressure/volume loop method for example), this methodology was in line with what we needed.

PUBLICATION n° 1

LONGITUDINAL ASSESSMENT OF HEART FUNCTION OF ZSF1 RATS BY ECHOCARDIOGRAPHY

Introduction

Afin de nous permettre de tester les effets potentiels de composés sur la fonction cardiaque, nous nous devions d'évaluer son évolution au cours de l'âge chez le rat ZSF1, ce qui jusqu'alors n'avait pas été effectué. Pour cela, nous avons évalué les fonctions diastolique et systolique des rats ZSF1 de manière longitudinale par l'utilisation de l'échocardiographie, méthode non invasive aussi utilisée chez l'Homme, à 12, 18, 24, puis 45 semaines. Le suivi de la fonction systolique s'est fait par la mesure de la fraction d'éjection, et le suivi de la function diastolique par le calcul du rapport E/A. En parallèle, une seconde cohorte de rats ZSF1 nous a permis d'estimer la sensibilité cardiaque à l'ischémie par la méthode de perfusion rétrograde de Langendorff à 12, 18, et 24 semaines.

Les rats ZSF1 ont été décrits comme étant modérément hypertendus. Nous avons donc mesuré la pression artérielle de ces animaux, également à 12, 18, 24, et 45 semaines. Nous avons émis l'hypothèse que la mise en place progressive du syndrome métabolique ainsi que le développement d'une hypertrophie ventriculaire gauche pourraient engendrer une baisse de la fonction cardiaque chez le rat ZSF1. Nous avons de plus évalué l'impact du syndrome métabolique sur la structure d'autres organes susceptibles d'être affectés. Le but de ces travaux fut de déterminer si la fonction cardiaque des rats ZSF1 se dégraderait avec l'âge, et dans quelle mesure, afin de mettre en évidence l'âge auquel l'effet protecteur de composés sur la fonction cardiaque pourrait être le mieux évalué.

Introduction

In order to be able to test the potential effect of compounds on heart function, we first needed to evaluate how the cardiac function evolved with aging in the ZSF1 rat. If the cardiac function and performance was steady and not declining along aging, we could not find the best age window to test putative cardio-protective effects of new compounds. Till now, no study had been done to evaluate evolution of this function with aging. To do so, we longitudinally evaluated both systolic and diastolic functions of male ZSF1 rats by the non-invasive echography technique, starting from a young adult age till an advanced age. The chosen time points were 12, 18, 24, and finally 45 weeks of age. Ejection fraction was measured to monitor systolic function and E/A ratio was calculated to assess the diastolic one. To confirm evolution of cardiac performance along aging, we used a second cohort of animals to assess the sensitivity to ischemia with the Langendorff methodology at 12, 18, and 24 weeks of age.

Knowing that these animals have been described as mild hypertensive, we measured blood pressure at 12, 18, 24 and 45 weeks of age. We hypothesized that development of metabolic syndrome along aging and development of left ventricle hypertrophy could participate to a decline in the cardiac function of ZSF1 rats. Additionally to cardiac function, we examined the impact of aging and progression of metabolic syndrome disease on detailed biochemical plasma and histological structural changes of other organs of ZSF1 rats. The final aim was to determine if cardiac function was impaired with age, and to which extend. Based on our data, we wished to define the right time-frame suitable to evaluate cardio-protective effects of compounds.

Experimental study 1 (*Journal of Experimental and Applied Animal Science*, 2013, 1(1):125-139)

Longitudinal assessment of heart function of ZSF1 rats by echocardiography

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Key words: Blood pressure; Echocardiography; Ischemia; Metabolic syndrome; ZSF1 rat model.

Running head: Heart function of ZSF1 rats

Aim: Development of Type 2 Diabetes (T2D) is associated with cardiac comorbidities linked to micro- and macro-vasculature defects. The aim of this work was to evaluate age-dependent changes in heart function by echocardiography in ZSF1 rats (Zucker fatty/Spontaneously hypertensive heart failure F1 hybrid), a model of metabolic syndrome.

Methods and Results: Male ZSF1 rats were studied at 12-18-24-45-57 weeks (W) of age in comparison to age-matched male lean ZSF1 rats. Left ventricular (LV) dimensions were measured by echocardiography in parasternal short axis view and M-mode to calculate Ejection Fraction (EF) and Fractional Shortening (FS). E and A waves were measured in parasternal four-chamber view (B-mode). Blood pressure (BP) was monitored by tail-cuff, cardiac structures by histology and sensitivity to ischemia by Langendorff method.

At all ages, lean ZSF1 and ZSF1 rats displayed moderate hypertension. ZSF1 rats were heavier (+47.4% body weight (BW) at 45W); their left ventricular mass was similar to lean at 12W but increased by +46.4% at 45W. ZSF1 rats had lower EF and FS than lean rats from the age of 24W (respectively -5% and -10%), and E/A wave ratio was lowered by 24% at 45W. Moreover, their hearts were more sensitive to ischemia. Additionally ZSF1 rats exhibited deterioration mainly on kidney and heart structure along aging.

Conclusion: With age, ZSF1 rats exhibit cardiac hypertrophy, deterioration of diastolic and systolic functions, and increased sensivity to ischemia. ZSF1 rats is a promising model for evaluating effects of drugs on cardiac function by echocardiography in the context of metabolic syndrome.

Introduction

T2D is a chronic disease leading to a shortening of life expectation of about 10 years. It is recognized by the World Health Organization as a global epidemic, affecting 311 million people in the world (American Diabetes Association 2011). Associated to T2D are co-morbidities such as hypertension, heart failure, cardiomyopathy and renal complications. There is an unmet need for medication curing or preventing the development of T2D-related co-morbidities, and especially involving cardiovascular and renal systems (Grundy et al. 2004; Grundy 2008).

In order to evaluate the efficacy of new anti-diabetics to prevent, slow or reverse deterioration of cardiovascular and renal functions, animal models combining insulin resistance, hypertension, cardiac dysfunction and diabetic nephropathy are required. Although several genetically (Zucker Diabetic Fatty, ZDF or Goto-Kakizaki, GK rats) or chemically modified rat (with Streptozotocin) models of T2D are available, they develop too rapidly towards overt diabetes without displaying the associated co-morbidities that develop concomitantly in human beings. As a consequence, the absence of complex models of T2D is a limitation for characterizing efficient new therapeutics. The ZDF rat model for example, develops features of metabolic syndrome, as found in human, like obesity, glucose intolerance, hyperglycemia, hyperinsulinemia, β-cells dysfunction and hyperlipidemia (Tofovic and Jackson 2003). However, this model does not develop hypertension with aging (Prabhakar et al. 2007). Furthermore, ZDF rats develop mild nephropathy with hydronephrosis (Marsh et al. 2007) which is not representative of the human diabetic population wherein glomerular and mesangial expansion, lesions of the tubules, and decrease in the filtration rate are found (Pollock and Carmines 2008; Remuzzi et al. 2006). GK rats belong to a strain characterized by absence of obesity and characterized by marginal glucose intolerance, reduction of β-cell (islets) mass without developing marked hyperglycemia (Movassat et al. 1997; Ling et al. 1998). Despite few signs of metabolic disturbances, GK rats are characterized by absence of obesity, by glomerulopathy and glomerular basal membrane thickening (one of the features observed in diabetic nephropathy). Spontaneously Hypertensive Rats (SHR) are strongly hypertensive and hypertriglyceridemic rodents

although without T2D, glucose intolerance or metabolic syndrome. SHR develop hypertension prior to exhibiting albuminuria or proteinuria and developing kidney damage (Koletsky 1973; Michaelis et al. 1986). The SHHF (Spontaneously Hypertensive Heart Failure) rat model develops hypertension leading to congestive heart failure (Heyen et al. 2002) although with a much milder metabolic syndrome than the recently developed hybrid ZSF1 rat model (Tofovic and Jackson 2003). The ZSF1 rat model originated from crossing lean female ZDF (ZDF +/fa) with lean SHHF male (SHHF/Mcc, $+/fa^{cp}$). This breeding led to the ZSF1 rat strain (fa/fa^{cp}), characterized by metabolic syndrome. This model is characterized by 2 mutations on the leptin receptor *fa* discovered by Zuckers in 1961, and *cp* described by Koletsky, which leads to leptin resistance (and not leptin deficiency) responsible for obesity as in humans (Schwartz et al. 2000). The ZSF1 model combines insulin resistance, mild hypertension, diabetic nephropathy and obesity, all features that are common in complications of T2D (Joshi et al. 2009; Tofovic and Jackson 2003). However it has also been reported that hypertension and T2D are associated with impaired LV diastolic function, independent of BW (Russo et al. 2010).

Till now, most of the published studies on the ZSF1 model mainly explored characteristics of metabolic changes (Tofovic and Jackson 2003) and to a certain extent on renal structure and function (Tofovic et al. 2000). None of them evaluated the longitudinal evolution of the cardiac function by echography in this animal model. In the present study, we aim at evaluating the cardiac function of male ZSF1 rats versus lean ZSF1 rats with the non-invasive echocardiography methodology at 12- 18- 24- and 45W. Echocardiography is a useful non-invasive tool to longitudinally evaluate changes in cardiac function in the same cohort of animals. The same approach is used in humans and allows depicting cardiac function changes in real time and in a quick manner (Scherrer-Crosbie and Thibault 2008). According to the recommendations of the Journal of the American Society of Echocardiography, one of the parameters to be measured in clinical trials to assess systolic function is EF, while the E/A ratio is a good indicator for diastolic function in human beings (Gottdiener et al. 2004). In humans, it is known that EF can be kept unchanged in spite of diastolic

dysfunction (Fiorini et al. 2004). These parameters are also recommended to assess both systolic and diastolic functions in preclinical studies (Hanton et al. 2008). Additionally to cardiac function, we examined the impact of aging and progression of metabolic syndrome disease on detailed structural changes of other organs of ZSF1 rats.

Material and methods

Animals

Male ZSF1 [(OB) Gmi Crl-fa/fa] and their lean counterparts [(OB) Gmi Crl-fa/_] were ordered at the age 10W from Charles River U.S. and directly fed a special diet PURINA PMI 5008 (Ssniff R/M-H, 10 mm). Rats were housed 4 per cage (Tecniplast®, Eurostandard Type IV, model 1354G, size: 595mm x 380mm x 200mm) at approximately 21°C and 55–65% relative humidity, and had *ad libitum* access to food and water. Light cycle was from 6 a.m. to 6 p.m.

Experimental procedures were performed under an animal research protocol (ARP) authorization to F. Hoffmann-La Roche by the Swiss Cantonal Veterinary Office Basel-Stadt and in accordance with guidelines of the Association for the Assessment and Accreditation of Laboratory and Animal Care (AAALAC # Unit Number: 001057) and appropriate federal, state and local guidelines.

Blood sampling methods and General metabolic parameters assessment

BW was recorded regularly along the aging period. At 12, 18, 24 and 45W, fasting blood glucose (FBG, 16h overnight fasting) was measured with Accuchek Aviva® portable system (Roche Diagnostics GmbH, Germany). Blood was collected from anaesthetized rats (isoflurane) by retrobulbar methodology (200µl of blood with EDTA) and dispatched into tubes coated with EDTA, containing 1% aprotinin (to inhibit protein degradation) and kept on ice. Animals were carefully monitored both before and after blood sampling and no signs of distress or complications were observed. Tubes were then centrifuged (10 000 RPM for 15 min at 4°C) and plasma was aliquoted in different tubes or 96-well plates for analysis. Blood samples were taken under non fasting conditions

in the morning 2h following switch from dark to light cycle and to assess glucose levels. Glucose and HbA1c (not shown) were measured in whole blood, while plasma was used for analysis of creatinine and other metabolic parameters (not shown).

Blood pressure monitoring

Systemic arterial BP measurements were performed non-invasively in conscious animals by using tail-cuff method, based on the Volume Pressure Recording technology (Model CODA; Kent Scientific Corporation, USA) (Ikeda et al. 1991). Systolic (SAP), diastolic (DAP), and mean (MAP) arterial pressures were measured 2 hours after the animals have been acclimated to a dedicated room heated at 28°C. The data were saved automatically on a spreadsheet through a computer interface after automatic validation out of twenty measurements by the system (exclusion criteria: shape of the curve with only one inflexion point) and a manual review (exclusion criteria: volume ≥30IU).

Echographic assessment of cardiac structure and function

Cardiac function was measured non-invasively by echocardiography using a Sequoia Acuson 512 system (Siemens AG, Germany) coupled to a 15L8 linear scan head. Rats were anesthetized with Isoflurane/O2 (induction 5% isoflurane, then 2%); the air was humidified to avoid dryness of the respiratory tract during the procedure. The whole intervention didn't exceed 25 minutes. Isoflurane was chosen among the existing anesthetics because it was shown to alter the least cardiac function (especially on the E and A waves) (Ling et al. 1998). Nevertheless, we are aware that isoflurane directly depresses myofilament contractility by decreasing Ca^{2+} responsiveness (Del Turco et al. 2013). Body temperature was monitored with a rectal temperature-probe thermometer (model TCAT-2LV Controller, Physitemp Instruments Inc., Clifton, USA) and maintained at 37°C by a coupled heating pad.

An in-house ECG system was coupled to the echography system to measure heart rate, which enabled us to monitor the stability of anesthesia throughout the intervention.

The echocardiography examination was performed according to the following protocol: In M mode and parasternal short axis view, the principal left ventricular dimensions were recorded, e.g. left ventricular internal diameter in diastole and systole (LVIDd, LVIDs), left ventricular posterior wall in diastole and systole (LVPWd, LVPWs), interventricular septum in diastole and systole (IVSd, IVSs). In Doppler mode and apical 4 chambers view, E and A waves were recorded (Fig. 1).

Each exam was performed in triplicate and measurements were done on 3 different pictures using the Showcase software (Trilium Technology Inc., USA). An average of the 3 measurements was done to increase the reliability of the data. Two well-trained echo performers generated the cardiac images. Prior to this investigation, reproducibility/repeatability of the investigation by each experimentator was assessed using wild type rats and ZSF1 obese rats. Similarly, we assessed the inter-experimentator variability.

Left ventricular mass (LV mass) was calculated according to the following corrected formula adapted to rodents:

$1.05x((LVID_d+LVPW_d+IVS_d)^3 - LVID_d^3) \times 0.8$ (Reffelmann and Kloner 2003). EF, FS and E/A ratio were calculated to assess the systolic and diastolic functions.

Sensitivity to ischemia assessed by langendorff method

In a separate age-matched cohort of ZSF1 rats (12W, 18W and 24W), buprenorphin (0.03mg/kg) and heparin (500 U.I.) were administered by s.c. injection ~30 min before anesthesia with thiobutabarbital i.p. (120mg/kg). Following intubation by tracheotomy, ZSF rats were ventilated with a rodent respirator (TOPOTM Dual Mode Ventilator, Kent Scientific Corporation, USA) at a pressure of 16 cm H2O delivered at 40 breaths / min. A median thoracotomy was performed, the ribs were retracted and the heart was exposed. The pericardium was removed and the ascending aorta cleared of all

connective tissue. The inferior vena cava was then clamped and an incision was made in the aorta. A steel cannula connected to the Langendorff system by a tygon tube was quickly inserted through the incision and then secured with a preplaced suture. The retrograde perfusion was established with modified Krebs-Henseleit solution (NaCl 118 mmol/L, KCl 4.7 mmol/L, $MgSO_4$ 1.64 mmol/L, KH_2PO_4 1.18 mmol/L, $CaCl_2$ 1.2 mmol/L, glucose 11 mmol/L, Na pyruvate 2 mmol/L+ 0.5 mmol/L lactate).

The heart was then transferred to the Langendorff system and perfused at a constant pressure of 130 mmHg. The hearts were allowed to stabilize for 20 min, perfused with modified Krebs-Henseleit solution for 20 min to determine baseline parameters. The hearts were then subjected to a global no flow ischemia for 40 min followed by 45 min of reperfusion.

Event marker was set appropriate for manipulation along the experiment. Perfusion pressure (PP), left ventricular derived pressure (LVdP), contractility (dLVP/dTmax), heart rate (HR) and coronary perfusion flow (CF) were simultaneously recorded with a computer data acquisition system during the entire experiment. Among all the cardiac parameters recorded, LVdP and contractility were reported in this study.

Time to Contracture (TTC) was evaluated during the ischemic period and defined as the duration between occlusion of coronary flow and start of ischemia-induced left ventricular contraction. The TTC was determined as time (minutes) during global ischemia necessary to observe the first increase of 0.5 mmHg in Left Ventricular End Diastolic Pressure (LVEDP).

Histology

At necropsy, kidney, heart, pancreas and liver were harvested and immediately immersed in 10% neutral buffered formalin for maximum 24h. Eyes were harvested and fixed in Davidson's fluid. After being rinsed, the tissues were embedded in paraffin blocks and slices of 4µm were prepared that were stained with standard hematoxylin eosin stain (HE; all tissues) and Periodic acid-Schiff reaction (PAS; kidney only). Heart was stained with toluidine blue for mast cell infiltration determination.

Scoring system for kidney structures changes was established as follows: (glomerular sclerosis and proteinaceous fluid)

0 (no change)
1 (minimal; less than 10 elements of a structure affected)
2 (slight; between 10 and 20 elements of a structure affected)
3 (moderate; between 20 and 50 less than 10 elements of a structure affected)

Cardiomyocyte diameter was measured from 10 cardiomyocytes per animal. Phosphotungstic acid hematoxylin (PTAH) that stains fibrins and erythrocytes in blue was used for evaluating cardiomyocyte striation.

Statistical analysis

Parameters measured in ZSF1 rats were compared to age-matched lean ZSF1 at each age of interest 12W, 18W, 24W and 45W. Fisher test was used to test homoscedasticity. For the metabolic and echocardiographic parameters, a 2-way ANOVA was used (§ p (group*age) <0.05), then a Student T-test for comparison at each age was performed (*p≤0.05; **p≤0.01; ***p≤0.001). For the Langendorff parameters, Student T-test was used for comparison at each age.

Results

General metabolic parameters

As displayed in Table 1, 12W-old ZSF1 rats already significantly exhibit higher BW and FBG as compared to age-matched lean ZSF1. FBG measured in ZSF1 rats constantly increased along aging from 12W to 24W. Hyperglycemia defined as elevated FBG (FBG>6.1mM) (American Diabetes Association 2011) was reached at the age of 18W. At the age of 45W, FBG measured in ZSF1 rats showed a tendency to decline. In contrast, lean ZSF1 displayed normal FBG level between 4 to 5mM from 12W to 45W. ZSF1 and lean rats constantly gain BW during their development. ZSF1 rats

became more obese than age-matched lean ZSF1 rats counterparts (by 31% to 47% at 12W and 45W of age, respectively).

Blood pressure along aging in ZSF1 rats

MAP, SAP and DAP of ZSF1 and lean ZSF1 rats didn't change with aging (Fig. 2). MAP measured by the tail cuff non-invasive method was already elevated at 12W for both groups, compared to wild type and non-hypertensive rodent models (153±6 mm Hg in ZSF1 and 132±9 mm Hg in lean ZSF1 rats). MAP was slightly but not significantly higher in ZSF1 rats at 12W (153±6 vs. 132±9 mmHg in lean ZSF1), 18W and 24W (138±6 vs. 125±6 mmHg in lean ZSF1), then similar than lean at 45W (154±5 vs. 151±7 mmHg in lean ZSF1).

Change in heart mass of ZSF1 rats evaluated by Echography

Structure

LV mass increased with age in both ZSF1 rats and lean rats (Fig. 3), in correlation with the increase in BW (Table 1). The increase in LV mass with age was higher in ZSF1 than in lean (p interaction < 0,05). At 12W, and despite the difference in BW (+114g in ZSF1 rats versus baseline, corresponding to 31% BW vs lean rats), no difference was found in the LV mass between ZSF1 and lean animals (1.06±0.03g vs 1.08±0.08g). At 18W, LV mass was increased in ZSF1 rats compared to lean (1.46±0.06g vs 1.26±0.11g, +16% of LV mass for the ZSF1 rats vs lean) in spite of a comparable difference in BW to 12W (164g at 18W, +37.1% of BW for ZSF1 rats vs lean). The same was observed in the rats at the age of 24W and 45W. A significant increase in LV mass in the ZSF1 rats compared to lean rats was found, especially at the endpoint where the highest difference was seen (2.02±0.08g vs 1.38±0.11g, +46.4% of LV mass for the ZSF1 rats vs lean ZSF1; difference of BW of 278g at 45W, +47.4% of BW for the ZSF1 rats vs lean ZSF1). Ratio LV mass/BW was higher in lean ZSF1 rats compared to age-matched ZSF1 rats until the age of 24W. No more difference was found

at 45W. Finally, despite higher increase in BW, the LV Mass/BW ratio tended to increase with age in ZSF1 rats and to decrease in lean rats (Fig. 3).

Increase in LV mass towards progressive hypertrophy correlates with changes in the left ventricular wall dimensions (Table 2). As an example, posterior wall LVPWd was already slightly higher in ZSF1 rats at 18W and 24W and significantly higher at 45W, as the IVS and LVID measured during the diastole. The same statement can be done for LVPWs, LVIDs and IVSs.

Changes in Cardiac Function of ZSF1 rats

Systolic and diastolic functions evaluated by echocardiography

EF and FS were constant in lean ZSF1 rats along aging as shown in Fig. 4. ZSF1 rats had significant lower EF and FS than lean rats from the age of 24W and persistent to 45W (respectively 80±1 vs. 84±1% for EF, and 42±1 vs. 46±1% for FS, at 45W).

E, A waves and E/A ratio were assessed from the age of 18W till 45W. From that stage, E/A ratio decreased in ZSF1 rats compared to age-matched lean rats. At 45W, E/A ratio was significantly reduced in ZSF1 rats compared to lean rats (by 24%, Fig. 5).

Contractility and Sensitivity to ischemia assessed by Langendorff

Basal cardiac performance was monitored ex vivo during retrograde perfusion of the excised heart. ZSF1 rats were characterized by significantly increased LVdP and contractility than age-matched lean rats at each age of interest (12, 18 and 24W) (Fig. 6). ZSF1 rats were more susceptible to ischemia than age-matched lean rats from 18W as depicted by the reduction of TTC (Fig. 6)). During reperfusion phase, ZSF1 rats recovered at around 35% of their LVdP while lean rats recovered at up

to 65% at the age of 12, 18 and 24W. Whole heart contractility measured at 12W-24W and right after reperfusion reached 41-45% in heart of ZSF1 rats versus 67-70% in lean age-matched rats.

Impact of aging on heart structure and other organs (Liver, pancreas, kidney and eyes)

Heart

In the heart, minimal focal to multifocal inflammatory infiltrates partly with cardiomyocyte degeneration and interstitial fibrosis were present in individual animals. In ZSF rats at the age of 24 and 57 weeks, the incidence and severity of the lesion were increased. In addition, ZSF rats at the age of 57 weeks exhibited cardiomyocyte hypertrophy (data not shown).

Liver

In the liver, minimal multifocal microgranulomas, marked to severe hepatocellular glycogen storage, minimal to slight centrilobular hepatocellular vacuolation and multifocal collagen I deposits were observed (not shown).

Pancreas

In the pancreas, slight to moderate cellular hypertrophy and vacuolation of islet cells, moderate to marked decreased roundness of islets, minimal increased extracellular matrix in islets and minimal focal atrophy of exocrine pancreas (in 3 of 12 animals) were observed (not shown).

Kidney

Histological examination of the kidney of ZSF1 rats over time showed increase in severity (Fig. 7a and Fig. 7b) and incidence of the kidney lesions (glomerulosclerosis, thickening of basement

membranes, interstitial inflammation and fibrosis and tubular dilation with luminal proteinaceous fluid, Fig. 8 and Fig. 9). In contrast, age-matched lean rats showed rare glomerulosclerosis and tubular dilation only.

Eyes

Only minimal cataract (in 1 of 12 animals) was observed in ZSF1 rats (not shown).

Discussion

As reported in literature and according to their genetic background, male ZSF1 rats became progressively hyperglycemic compared to lean litter mates. ZSF1 rats were also characterized by constant BW increase along aging and rapidly developed obesity (Tofovic et al. 2000; Tofovic and Jackson 2003). In our work, we showed that male ZSF1 rats from 12W to 45W of age progressed constantly towards metabolic syndrome (i.e. development of hyperglycemia, maintenance of hypertension and development of obesity).

From 12W, lean ZSF1 and ZSF1 rats were already mild hypertensive without further elevation of blood pressure along aging as already shown in the literature (Tofovic et al. 2000). The slight upper blood pressure values measured in ZSF1 compared to lean rats might not be linked to the impairment of cardiac or renal function (Griffin et al. 2007). Impairment of endothelial function could be a consequence of long-term hyperglycemia and hyperlipidemia (gluco- and lipotoxicity) (Del Turco et al. 2013; Zhang et al. 2012) as we observed in this study. The coexistence of obesity with hyperglycemia and constant mild hypertension could contribute to morphological changes in the heart and other key organs sensitive to metabolic syndrome and hypertension. Then, these morphological changes observed in the heart of ZSF1 certainly contributed to cardiac functional changes observed in this model.

LV hypertrophy of ZSF1 rats is in correlation with the increase in cardiac wall dimensions measured in ZSF1 rats along aging, and compared to lean rats. LV hypertrophy observed in the ZSF1 rats led to functional changes as compared to lean ZSF1 rats: 1/ Systolic function, assessed by the measurement of both EF and FS, slightly decreased from 24W of age onwards although were still above 80% and 40%, respectively and weren't worsened further at 45W. 2/ Diastolic function was already impaired from 18W, while it was more drastically impaired at 45W. These changes support the hypothesis that the diastolic dysfunction occurs before the impairment of systolic function and develops along T2D progression, as reported in human beings (Fiorini et al. 2004; Stahrenberg et al. 2010). 3/ Increase in contractility and LVdP are concomitant to deterioration in E/A ratio that was measured at advanced age. Functional cardiac changes observed in ZSF1 rats during aging, paralleled structural lesions (inflammatory infiltrates, cardiomyocyte degeneration and interstitial fibrosis and cardiomyocyte hypertrophy) which incidence and severity increased with disease progression although remained minor to moderate (histological semi-quantitative analysis, data not shown). In the heart, minimal focal to multifocal inflammatory infiltrates partly with cardiomyocyte degeneration and interstitial fibrosis were present in individual animals. In ZSF rats at the age of 24W and 57W, the incidence and severity of the lesions were increased. In addition, ZSF rats at the age of 57W exhibited cardiomyocyte hypertrophy.

4/ After ischemia induced in perfused isolated heart (Langendroff method), the decrease in TTC observed in ZSF1 rats along aging compared to lean rats suggests a higher susceptibility to ischemia. During the reperfusion phase, heart's contractility was impaired in ZSF1 rats compared to lean rats. Those findings strengthen the impairment of heart of ZSF1 rat to ischemia/reperfusion as observed in T2D patients versus non-diabetics (Le Feuvre et al. 2011). Similarly, our data could translate the higher and susceptibility to congestive heart failure in ZSF1 rats as observed in T2D patients (Grundy et al. 1999). Langendroff method is a usefull tool applied to rodent models to evaluate pathogenesis of T2D and associated cardiovascular morbidity (Kristiansen et al. 2004; Tofovic and Jackson 2003). As reported for ZDF rats (Tofovic and Jackson 2003), metabolic and contractile

abnormalities are observed in ZSF1 rats. As opposed to ZDF and GK rats, ZSF1 rats are characterized by higher susceptibility and sensitivity to ischemia (Kristiansen et al. 2004; Wang and Chatham 2004). Discrepancy in heart function and sensitivity to ischemia between ZSF1 and ZDF or GK rats could be due to a more complex metabolic disorder that characterizes ZSF1 rats (metabolic syndrome versus T2D) and the presence of mild-to-severe hypertension.

In the ZSF1 rat model, aging also impacted other organs than the heart, such as liver, pancreas and kidney. In the liver, minimal multifocal microgranulomas, marked to severe hepatocellular glycogen storage, minimal to slight centrilobular hepatocellular vacuolation and multifocal Collagen I deposits were observed.

In the pancreas, slight to moderate cellular hypertrophy and vacuolation of islet cells, moderate to marked decreased roundness of islets, minimal increased extracellular matrix in islets and minimal focal atrophy of exocrine pancreas (in 3 of 12 animals) were observed. These data confirmed the metabolic syndrome state of ZSF1 rats. Histological examination of the kidney of ZSF1 rats over time showed increase in severity and incidence of the kidney lesions (glomerulosclerosis, thickening of basement membranes, interstitial inflammation and fibrosis and tubular dilation with luminal proteinaceous fluid). Significant changes in kidney structures suggested a worsening of kidney function in ZSF1 rats as previously reported by Tofovic and al. (2000). The extent of the kidney changes would suggest worsening of kidney function. A dedicated assessment of kidney function could be done by Glomerular Function Rate measurement or creatinine clearance determination and measurement of specific biomarkers in urine and plasma.

In this study, we report the changes in cardiac function in ZSF1 rats along aging and development of metabolic syndrome (T2D, hypertension) as already indicated in the literature (Tofovic et al. 2000). For the first time, we report the simultaneous changes in cardiac structure and function at early adult age in ZSF1 rats (before the age of 46W). The precision of the echocardiographic assessments

confirmed the adaptation of the heart structure (hypertrophy and increase in contractility) prior to cardiac function deterioration (impairment of diastolic and systolic functions). With echocardiography method, we were able to highlight the step-by-step changes in cardiac function despite absence of increase in blood pressure. We also demonstrated that the structural changes of the heart started relatively early in this model, since LV mass and LV wall dimensions already showed a trend towards hypertrophy at 18W.

In this study, we characterized impaired sensitivity of ZSF1 rats to ischemia. This new finding reinforces the fact that modifications of cardiac function in this metabolic syndrom rodent model are ongoing (may be worsened in older ages or with further development of hypertension).

Taking into consideration the progression and relative modest extent of cardiac changes, we believe that those adaptations are the beginning of a process. Actually, the study performed by Tofovic et al. showed that adult male ZSF1 rats (11 months of age, e.g. 46W old), were characterized by cardiac function intermediate to those measured in SHHF rats (Heyen et al. 2002; Tofovic and Jackson 2003) and SHR (Koletsky 1973; Michaelis et al. 1986). Additionally, the progressive stuctural damages observed in kidneys (glomerulosclerosis, thickening of basement membranes, interstitial inflammation and fibrosis and tubular dilation), as described by Tofovic et al. (2000), could support the progression/potential of ZSF1 rats to metabolic and cardio-renal disease model. Especially, the exacerbation of kidney score at advanced age (57W) confirmed the dramatic deterioration of kidney structures (and potentially function) of this animal model.

In conclusion, the functional cardiac changes observed in ZSF1 rat model evolution show that it could constitute a translational model to evaluate cardio protective impact of drugs. This model - associated with the use of echocardiography- is appropriate to assess efficacy of new protective drugs acting as intervention or prevention therapies. Using the echocardiography method allows longitudinal functional and structural measurements that can depict early changes as triggers of cardiac remodeling. Finally, the development of cardiac, renal and metabolic pathologies of the ZSF1

rat model might be further exacerbated (faster and greater extent of the damages) by impairment of kidney function via induction of kidney hyper filtration (surgical approach) or via the increasing in blood pressure (pharmacological approach). This could be an alternative to develop cardio-renal metabolic syndrome model in a shorter term than within 45-57W of age.

Funding

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Conflict of interest

None declared.

References

1. American Diabetes Association. Diagnosis and classification of diabetes mellitus (2011) Diabetes Care 34 (Suppl 1): S62-S69.

2. American Diabetes Association. Standards of Medical Care in Diabetes–2011 (2011) Diabetes Care 34 (Suppl 1): S11–S61.

3. Del Turco S, Gaggini M, Daniele G, Basta G, Folli F, Sicari R, Gastaldelli A (2013) Insulin resistance and endothelial dysfunction: a mutual relationship in cardiometabolic risk. Curr Pharm Des 19(13):2420-31.

4. Ding W, Li Z, Shen X, Martin J, King SB, Sivakumaran V, Paolocci N, Dong Gao W (2011) Reversal of Isoflurane-Induced Depression of Myocardial Contraction by Nitroxyl via Myofilament Sensitization to Ca^{2+}. J Pharmacol Exp Ther 339(3): 825-831.

5. Fiorini G, Scotti LA, Parmigiani ML, Ferrari M, Pezzoli P, Bignotti G (2004) An echocardiographic study of left ventricular diastolic function in patients with diabetes mellitus type 2. G Ital Cardiol 25(1): 17-25.

6. Gottdiener JS, Bednarz J, Devereux R, Gardin J, Klein A, Manning WJ, Morehead A, Kitzman D, Oh J, Quinones M, Schiller NB, Stein JH, Weissman NJ (2004) American Society of Echocardiography recommendations for use of echocardiography in clinical trials. J Am Soc Echocardiogr 17(10): 1086-1119.

7. Griffin KA, Abu-Nasser M, Abu-Amarah I, Picken M, Williamson GA, Bidani AK (2007) Dynamic blood pressure load and nephropathy in the ZSF1 (fa/fa^{cp}) model of Type 2 diabetes. Am J Physiol 293: F1605-F1613.

8. Grundy SM, Benjamin IJ, Burke GL, Chait A, Eckel RH, Howard BV, Mitch W, Smith SC Jr, Sowers JR (1999) Diabetes and Cardiovascular Disease: A Statement for Healthcare Professionals. From the American Heart Association. Circulation 100:1134-1146.

9. Grundy SM, Brewer HB Jr, Cleeman JI, Smith SC Jr, Lenfant C (2004) Definition of Metabolic Syndrome: Report of the National Heart, Lung, and Blood. Circulation 109:433-438.

10. Grundy SM (2008) Metabolic Syndrome Pandemic. Arterioscl Throm Vasc 28(4): 629-636.

11. Hanton G, Eder V, Rochefort G, Bonnet P, Hyvelin JM (2008) Echocardiography, a non-invasive method for the assessment of cardiac function and morphology in preclinical drug toxicology and safety pharmacology. Expert Opin Drug Metab Toxicol 4(6): 681-696.

12. Heyen JR, Blasi ER, Nikula K, Rocha R, Daust HA, Frierdich G, Van Vleet JF, De Ciechi P, McMahon EG, Rudolph AE (2002) Structural, functional, and molecular characterization of the SHHF model of heart failure. Am J Physiol 283(5): H1775-H1784.

13. Ikeda K, Nara Y, Yamori Y (1991) Indirect systolic and mean blood pressure determination by a new tail cuff method in spontaneously hypertensive rats. Lab Anim, 25(1):26-9.

14. Joshi D, Gupta R, Dubey A, Shiwalkar A, Pathak P, Gupta RC, Chauthaiwale V, Dutt C (2009) TRC4186, a novel AGE breaker, improves diabetic cardiomyopathy and nephropathy in Ob-ZSF1 model of Type 2 Diabetes. J Cardiovasc Pharm 54(1): 72-81.

15. Koletsky, S (1973) Obese spontaneously hypertensive rats—a model for study of atherosclerosis. Exp Mol Pathol 19: 53-60.

16. Koletsky S (1975) Pathologic findings and laboratory data in a new strain of obese hypertensive rats. Am J Pathol 80, 129-142.

17. Kristiansen SB, Løfgren B, Støttrup NB, Khatir D, Nielsen-Kudsk JE, Nielsen TT, Bøtker HE, Flyvbjerg A (2004) Ischaemic preconditioning does not protect the heart in obese and lean animal models of Type 2 diabetes. Diabetologia 47:1716–1721.

18. Le Feuvre C, Jacqueminet S, Barthelemy O (2011) Myocardial ischemia: a silent epidemic in Type 2 diabetes patients. Future Cardiol 7(2):183-90.

19. Ling ZC, Efendic S, Wibom R, Abdel-Halim SM, Ostenson CG, Landau BR, Khan A (1998) Glucose metabolism in Goto-Kakizaki rat islets. Endocrinology. 139(6):2670-5.

20. Marsh SA, Powell PC, Agarwal A, Dell'Italia LJ, Chatham JC (2007) Cardiovascular dysfunction in Zucker obese and Zucker diabetic fatty rats: role of hydronephrosis. Am J Physiol 293(1): H292-H298.

21. Michaelis OE 4th, Patrick DH, Hansen CT, Canary JJ, Werner RM, Carswell N (1986) Spontaneous hypertensive/NIH-corpulent rat. Animal model of human disease insulin-independent diabetes mellitus (type II). Am J Pathol 123:398-400.

22. Movassat J, Saulnier C, Serradas P, Portha B (1997) Impaired development of pancreatic b-cell mass is a primary event during the progression to diabetes in the GK rat. Diabetologia 40:916–925.

23. Plante E, Lachance D, Roussel E, Drolet MC, Arsenault M, Couet J (2006) Impact of anesthesia on echocardiographic evaluation of systolic and diastolic function in rats. J Am Soc Echocardiogr 19(12): 1520-1525.

24. Pollock JS and Carmines PK (2008) Diabetic nephropathy: nitric oxide and renal medullary hypoxia. Am J Physiol 294(1): F28-F29.

25. Prabhakar S, Starnes J, Shi S, Lonis B, Tran R (2007). Diabetic Nephropathy Is Associated with Oxidative Stress and Decreased Renal Nitric Oxide Production. J Am Soc Nephrol 18(11): 2945-2952.

26. Reffelmann T and Kloner RA (2003) Transthoracic echocardiography in rats. Evaluation of commonly used indices of left ventricular dimensions, contractile performance, and hypertrophy in a genetic model of hypertrophic heart failure (SHHF-Mcc-facp-Rats) in comparison with Wistar rats during aging. Basic Res Cardiol 98(5): 275-284.

27. Remuzzi G, Benigni A, Remuzzi A (2006) Mechanisms of progression and regression of renal lesions of chronic nephropathies and diabetes. J Clin Invest 116(2): 288-296.

28. Russo C, Jin Z, Homma S, Rundek T, Elkind MSV, Sacco RL, Di Tullio MR (2010) Effect of diabetes and hypertension on left ventricular diastolic function in a high-risk population without evidence of heart disease. Eur J Heart Fail 12(5): 454-461.

29. Scherrer-Crosbie M and Thibault HB (2008). Echocardiography in translational research: of mice and men. J Am Soc Echocardiogr 21(10): 1083-1092.

30. Schwartz MW, Woods SC, Porte, DJr, Seeley RJ, Baskin DG (2000) Central nervous system control of food intake. Nature 404(6778): 661-671.

31. Stahrenberg R, Edelmann F, Mende M, Kockskämper A, Düngen HD, Scherer M, Kochen MM, Binder L, Herrmann-Lingen C, Gelbrich G, Hasenfuß G, Pieske B, Wachter R (2010) Association of glucose metabolism with diastolic function along the diabetic continuum. Diabetologia 53(7): 1331-1340.

32. Tofovic SP, Kusaka H, Kost CK, Bastacky S (2000) Renal function and structure in diabetic, hypertensive, obese ZDFxSHHF-hybrid rats. Ren Fail 22(4): 387-406.

33. Tofovic SP and Jackson EK (2003) Rat Models of the Metabolic Syndrome. Meth Mol Med, 86: 29-46.

34. Wang P and Chatham JC. Onset of diabetes in Zucker diabetic fatty (ZDF) rats leads to improved recovery of function after ischemia in the isolated perfused heart (2004) Am J Physiol 286: E725–E7364.

35. Zhang H, Dellsperger KC, Zhang C (2012) The link between metabolic abnormalities and endothelial dysfunction in type 2 diabetes: an update. Basic Res Cardiol 107(1):237.

Figures and tables

Figure 1 **Different heart views by echocardiography in 45 week-old lean ZSF1 rat. a) Parasternal long axis view; b) Parasternal short axis view; c) Apical 4 chambers view. LV=left ventricle, RV= right ventricle, RA= right atrium, LA= left atrium, Ao= Aorta.**

Figure 2 **Blood pressures of lean ZSF1 and ZSF1 rats measured by the tail-cuff method along aging. Data are expressed as mean ± SEM; n=7.**

Figure 3 **Evolution of left ventricular mass along aging of lean ZSF1 and ZSF1 rats evaluated by echocardiography. Data are expressed as mean ± SEM; n=9.**

Figure 4 **Evaluation of the left ventricular systolic function: Ejection fraction and fractional shortening evolution in lean ZSF1 and ZSF1 rats. Data are expressed as mean ± SEM; n=9.**

Figure 5 **Evaluation of the left ventricular diastolic function. (a) E/A ratio evolution in lean ZSF1 and ZSF1 rats obtained by echocardiography. Data are expressed as mean ± SEM; n=9. (b, c) E wave and A wave, measured in 45W lean ZSF1 (b) and ZSF1 (c) rats by echocardiography in Doppler mode. E wave represents the passive filling of the left ventricle; A represents the active filling of the left ventricle, via left atrial contraction.**

Figure 6 **Langendorff parameters. (a) LVdP and contractility. Baseline cardiac parameters assessed ex vivo by Langendorff method prior to ischemia. (b) Time to contracture after ischemia, performed for 40 minutes. Data are expressed as mean ± SEM. N=8 per group.**

Figure 7 **Detailed (a) and summarized (b) kidney score in ZSF rats along aging. Comparison with lean rats.**

Figure 8 **Representative Kidney structures in ZSF1 along aging (PAS staining).**

Figure 9 **Representative Kidney structures in 57 week old ZSF1 rat showing nephropathy with glomerulosclerosis. Thickening of basement membranes (1), interstitial inflammation and fibrosis (2), tubular dilation with luminal proteinaceous fluid (3) (PAS staining).**

Table 1 **Body weight and fasting blood glucose values in lean ZSF1 and ZSF1 rats along aging. Data are expressed as mean ± SEM; n=9.**

Table 2 **Left ventricular dimensions of lean ZSF1 and ZSF1 rats measured along aging. Data are expressed as mean ± SEM; n=9.**

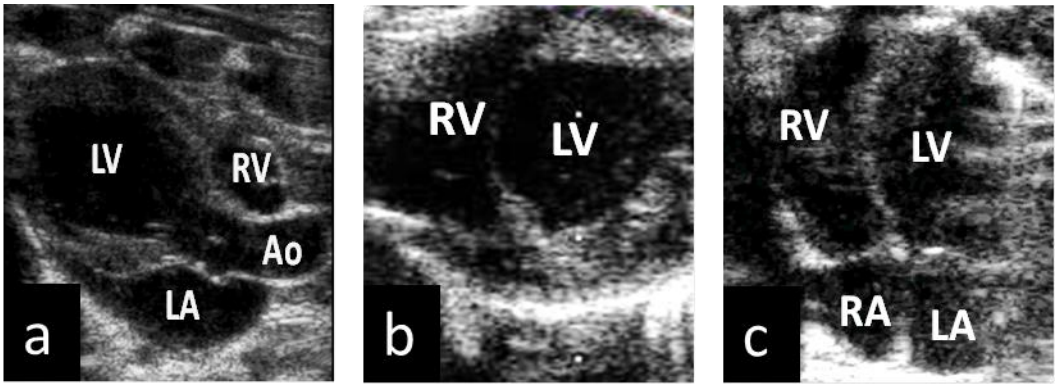
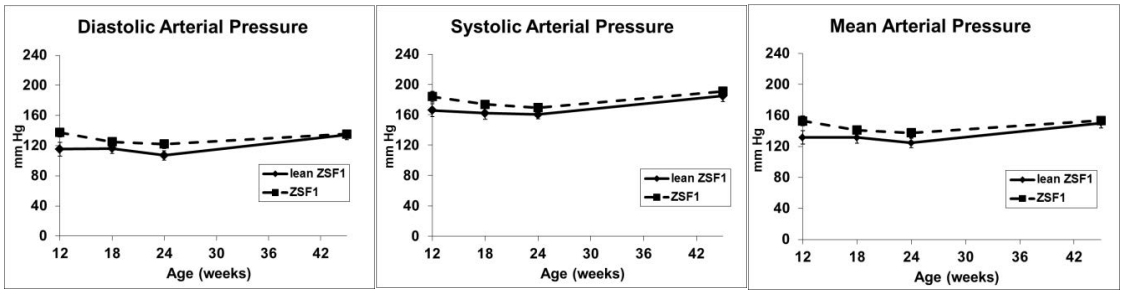


Figure 1



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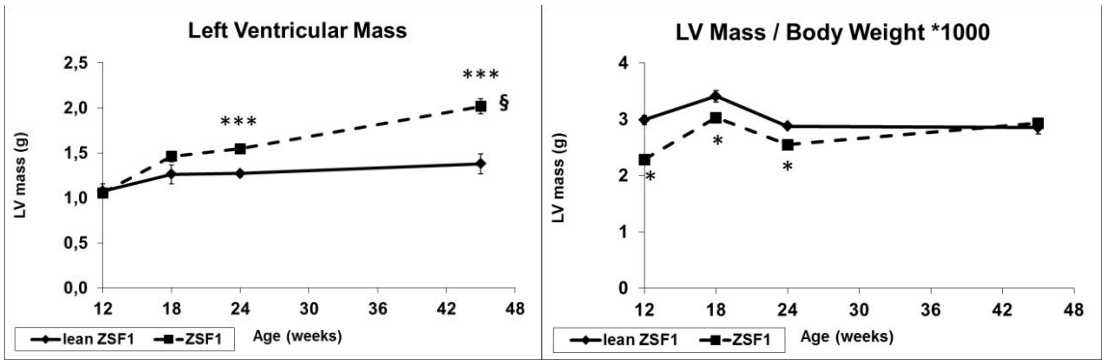

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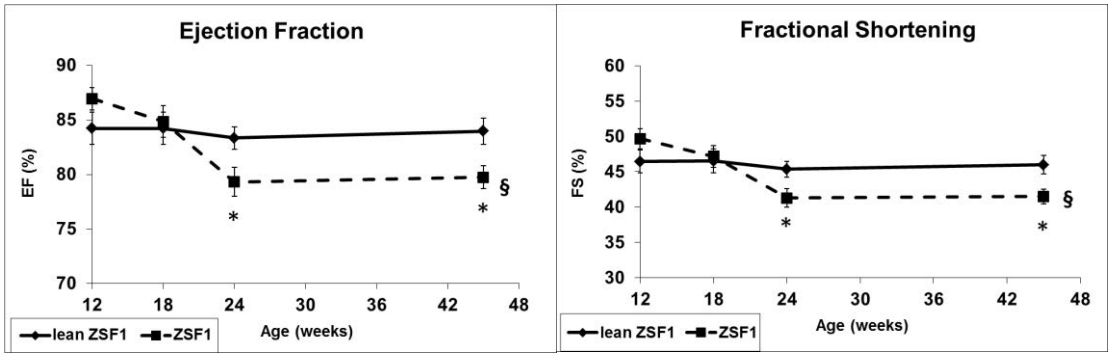

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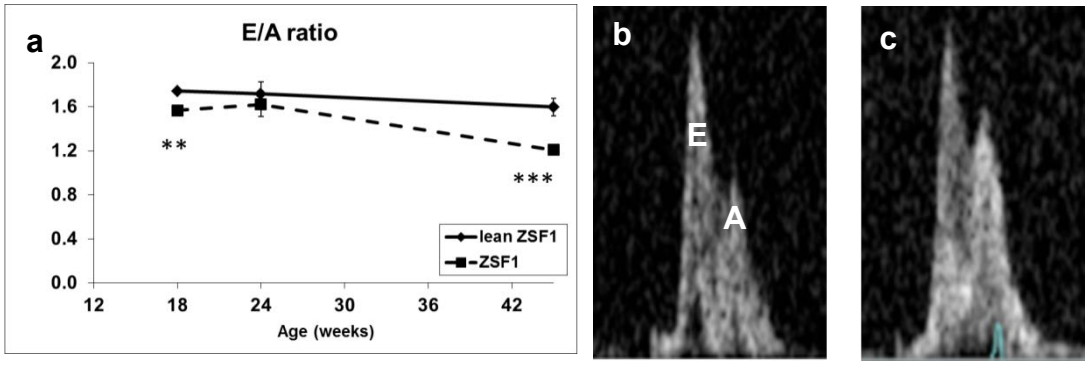

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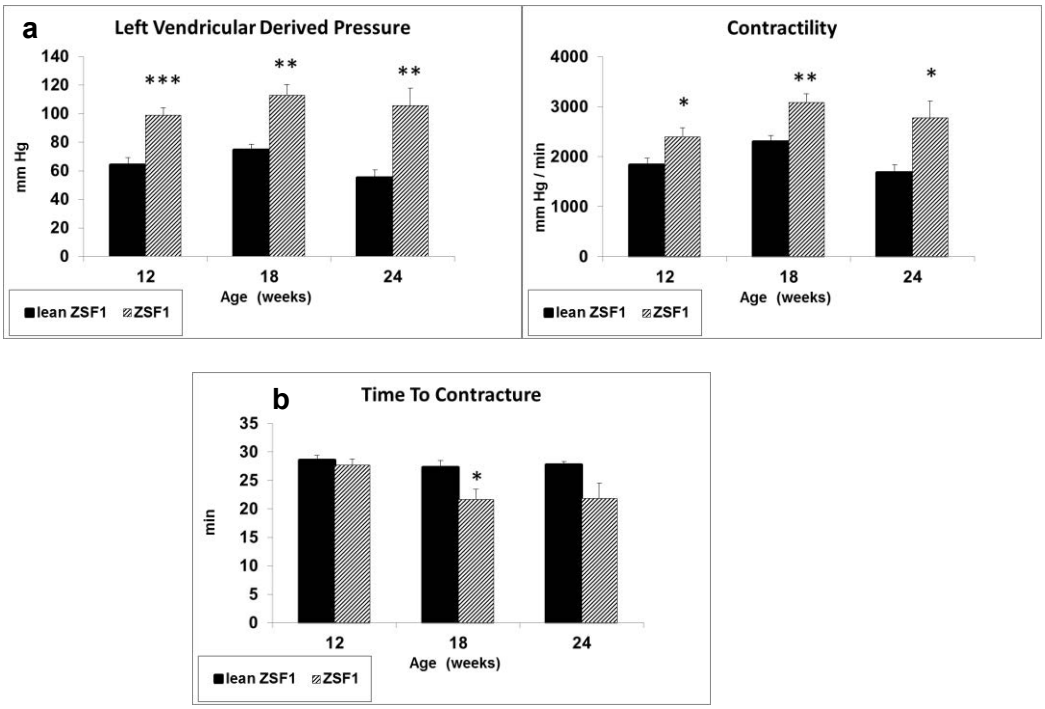
a
Left Vendricular Derived Pressure
mm Hg
140
120
100
80
60
40
20
0
***
**
**
12
18
24
Age (weeks)
lean ZSF1
ZSF1
Contractility
mm Hg / min
4000
3000
2000
1000
0
*
**
*
12
18
24
Age (weeks)
lean ZSF1
ZSF1
b
Time To Contracture
min
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18
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Age (weeks)
lean ZSF1
ZSF1

Figure 6

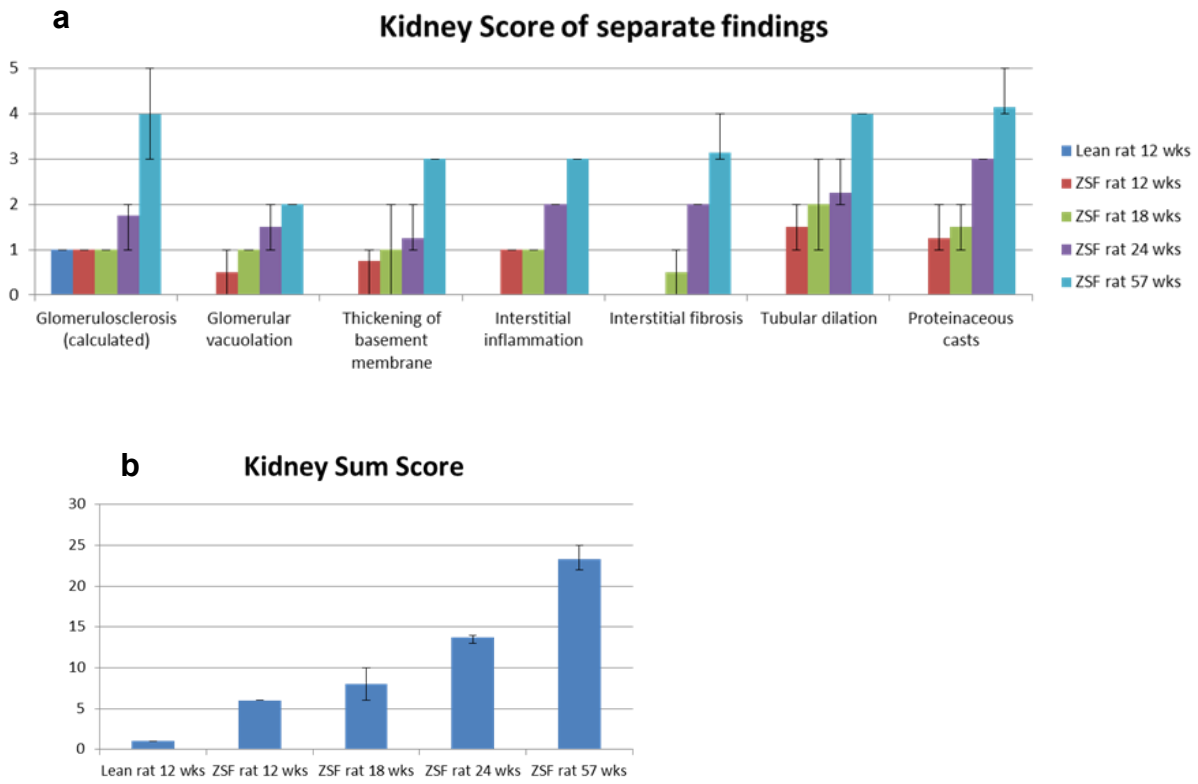
a
Kidney Score of separate findings
5
4
3
2
1
0
Glomerulosclerosis (calculated)
Glomerular vacuolation
Thickening of basement membrane
Interstitial inflammation
Interstitial fibrosis
Tubular dilation
Proteinaceous casts
Lean rat 12 wks
ZSF rat 12 wks
ZSF rat 18 wks
ZSF rat 24 wks
ZSF rat 57 wks
b
Kidney Sum Score
30
25
20
15
10
5
0
Lean rat 12 wks
ZSF rat 12 wks
ZSF rat 18 wks
ZSF rat 24 wks
ZSF rat 57 wks

Figure 7

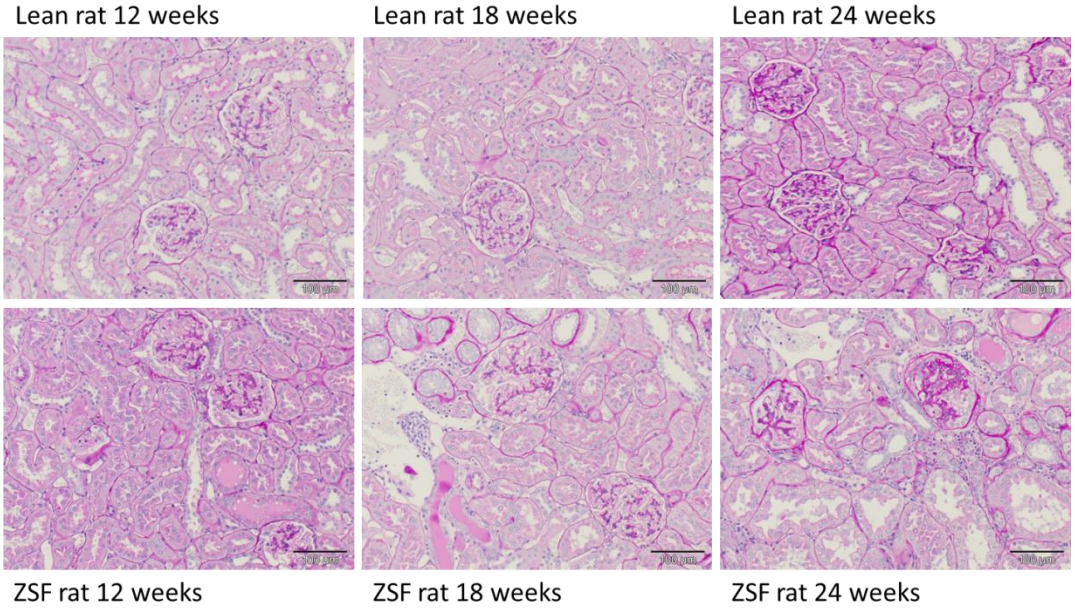

Figure 8

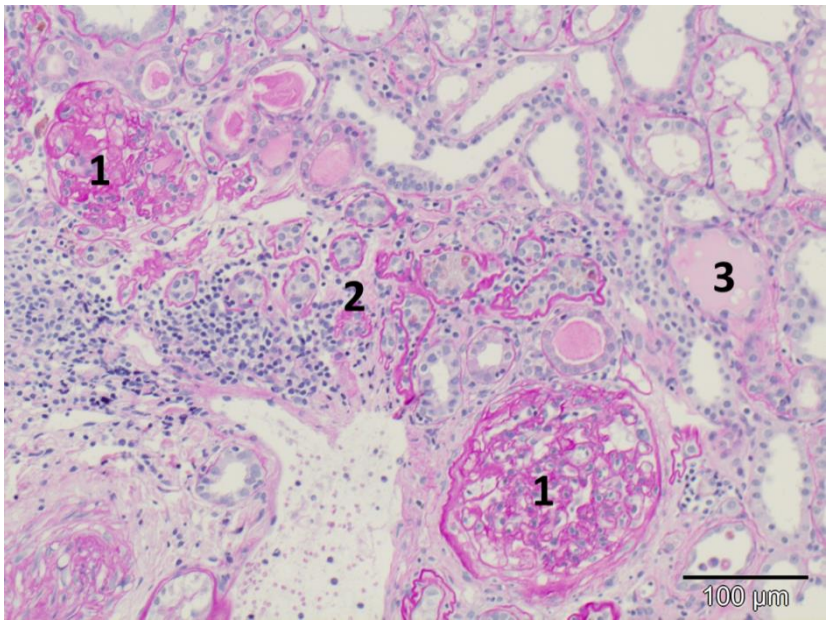

Figure 9

Table 1

		lean ZSF1	ZSF1
12 weeks	BW (g)	369 ± 8	483 ± 6 ***
	FBG (mM)	4.0 ± 0.1	5.7 ± 0.4 ***
18 weeks	BW (g)	442 ± 6	606 ± 7 ***
	FBG (mM)	3.8 ± 0.1	7.1 ± 0.6 ***
24 weeks	BW (g)	483 ± 6	688 ± 8 ***
	FBG (mM)	4.5 ± 0.1	9.0 ± 0.5 ***
45 weeks	BW (g)	587 ± 6	866 ± 20 *** §
	FBG (mM)	4.8 ± 0.1	6.8 ± 0.3 *** §

Table 2

	lean ZSF1				ZSF1			
Age (weeks)	12	18	24	45	12	18	24	45
IVS d cm	0.19 ± 0.01	0.19 ± 0.01	0.20 ± 0.00	0.20 ± 0.01	0.18 ± 0.00	0.19 ± 0.01	0.19 ± 0.00	0.22 ± 0.00 * §
LVID d cm	0.80 ± 0.01	0.82 ± 0.02	0.82 ± 0.01	0.88 ± 0.02	0.81 ± 0.01	0.88 ± 0.01 *	0.92 ± 0.03 **	0.95 ± 0.02 *
LVPW d cm	0.23 ± 0.02	0.25 ± 0.02	0.26 ± 0.01	0.24 ± 0.02	0.23 ± 0.01	0.27 ± 0.02	0.27 ± 0.02	0.31 ± 0.01 *
IVS s cm	0.33 ± 0.01	0.33 ± 0.01	0.34 ± 0.01	0.35 ± 0.01	0.34 ± 0.01	0.35 ± 0.01	0.33 ± 0.01	0.41 ± 0.03
LVID s cm	0.43 ± 0.02	0.44 ± 0.02	0.45 ± 0.01	0.46 ± 0.02	0.41 ± 0.02	0.47 ± 0.02	0.54 ± 0.03 **	0.55 ± 0.02 ** §
LVPW s cm	0.33 ± 0.02	0.36 ± 0.02	0.37 ± 0.02	0.36 ± 0.02	0.35 ± 0.01	0.40 ± 0.02	0.37 ± 0.01	0.41 ± 0.01 *

Discussion - Conclusion

Nous avons confirmé dans cette étude que le rat ZSF1 développait de manière progressive un syndrome métabolique au cours de l'âge. En effet, l'hypertension modérée est restée stable avec l'âge, l'obésité et l'hyperglycémie se sont quant à eux développées, en parallèle d'une hypertrophie ventriculaire gauche. De plus, la fonction diastolique s'est montrée altérée à partir de 18 semaines, et a continué à se dégrader jusqu'à 45 semaines, alors que la fonction systolique est restée préservée et supérieure à 80 %, à 45 semaines. Les données hstologiques ont mis en évidence le développement de faibles lésions au niveau du cœur et des autres organes jusqu'à 45 semaines. A un âge plus avancé, à 57 semaines, une hypertrophie des cardiomyocytes a été observée, et d'autres organes ont été impactés de manière significative par le syndrome métabolique tels que le pancréas, le foie ou encore les reins. Enfin, les cœurs des rats ZSF1 ont montré une plus grande susceptibilité à l'ischémie que ceux des animaux ZSF1-lean.

En conclusion, nous avons mis en évidence des adaptations structurales au niveau cardiaque (hypertrophie et augmentation de la contractilité), ayant lieu avant le développement d'une baisse modérée de la fonction cardiaque (diastolique) chez les rats ZSF1 adultes (à 45 semaines). L'échographie cardiaque nous a permis de suivre ce déclin au cours de l'âge, et le Langendorff de montrer une sensibilité à l'ischémie accrue chez le rat ZSF1. Ces changements montrent que ce modèle de rat ZSF1 pourrait constituer un bon modèle permettant d'évaluer l'effet protecteur de composés sur la fonction cardiaque. Cependant, le développement des alterations non seulement cardiaques, mais aussi rénales et métaboliques pourraient certainement être exacerbées en termes d'importance et de rapidité d'évolution, afin que ce modèle se développe en un temps plus court que 45 à 57 semaines, avec une défaillance cardio-rénale concommitante à l'évolution du syndrome métabolique. Pour cela, une hyperfiltration rénale (approche chirurgicale) ou une augmentation de la pression artérielle (approche hormonale) pourraient être envisagées.

Discussion - Conclusion

In this study, we showed that male ZSF1 rats from 12 weeks to 45 weeks of age progressed constantly towards metabolic syndrome. Indeed, hypertension was maintained, hyperglycemia increased with age as well as obesity. Increase in heart wall dimensions showed that left ventricular hypertrophy developed with age. In parallel, systolic function slightly decreased from 25 weeks of age, but was still higher than 80%, meaning that systolic function was maintained until 45 weeks of age. Diastolic function was impaired from 18 weeks of age, and declined further till 45 weeks of age. Histology showed that mild to moderate structural lesions developed with aging in heart and other organs, except at 57 weeks of age. At this more advanced age, cardiomyocyte hypertrophy was observed. Metabolic syndrome impacted others key organs such as pancreas, kidneys or liver. Finally, hearts of ZSF1 rats were more sensitive to ischemia as compared to those of lean littermates.

To conclude, we reported the simultaneous changes in cardiac structure and function at early adult age in ZSF1 rats (before the age of 46 weeks) with adaptation of the heart structure (hypertrophy and increase in contractility) prior to cardiac dysfunction (impairment of diastolic function). Moreover, although hypertension did not develop further along aging, echocardiography method allowed depicting the step-by-step changes in cardiac function. We also characterized impaired sensitivity of ZSF1 rats to ischemia. The functional cardiac changes observed in ZSF1 rat model evolution show that it could constitute a translational model to evaluate cardio protective impact of drugs. This model -associated with the use of echocardiography- is appropriate to assess efficacy of new protective drugs acting as (early) intervention or prevention therapies. However, the development of cardiac, renal and metabolic pathologies of the ZSF1 rat model might be further exacerbated (faster and greater extent of the damages) by impairment of kidney function *via* induction of kidney hyper filtration (surgical approach) or *via* an increasing in blood pressure (pharmacological approach). This could be an alternative to develop cardio-renal metabolic syndrome model in a shorter time-frame than 45-57 weeks of time, and with a greater extent.

2.5 ALTERATION OF RENAL FUNCTION BY UNILATERAL NEPHRECTOMY IN THE ZSF1 RAT MODEL

2.5.1 Renal function of ZSF1 rat

Only few publications have evaluated renal function of the ZSF1 rat. Tovovic *et al.* reported that ZSF1 rats displayed severe structural and functional kidney features, *i.e.* dilated tubules, glomerulosclerosis, mesangial expansion, arteriolar sclerosis, and marked urinary protein excretion. It was proposed that the ZSF1 rats probably died of end-stage renal failure at about a year of age (Tofovic *et al.* 2000). It was also shown that development of kidney disease was not in direct association with the development of systemic hypertension (Griffin *et al.* 2007). Moreover, it has been shown that early signs of renal disease, *i.e.* proteinuria, glomerular deposition of collagen IV, tubulointerstitial inflammation, renal hypertrophy and a decrease in GFR are already present at 8 weeks of age. These defects would be due to the developing metabolic syndrome status of the animal (Bilan *et al.* 2011). At 32 weeks of age, Bilan *et al*. showed a 25 fold increase in albuminuria as compared to lean littermates, but with a maintained kidney function assessed by GFR determination. This could be explained by an hyperfiltration induced by hyperglycemia as reported in many rat strains of diabetes (O'Donnell *et al.* 1988; Palm *et al.* 2001) or by hyperphagia that induces an increase in protein intake, associated with an increased renal blood flow (Woods 1993). In 2010, a classification for glomerular lesions for human type-1 and type-2 diabetic nephropathy was elaborated (Tervaert *et al.* 2010). Four stages have been defined and Bilan *et al.* stated that 20- to 44-week-old obese ZSF1 rats would develop class II glomerular lesions, *i.e.* with basement membrane thickening and mesangial expansion with mild to moderate glomerulosclerosis (5 to 30%). Moreover, ZSF1 rats have been shown to develop interstitial and vascular lesions typical of human diabetic nephropathy such as interstitial fibrosis and inflammation, tubular dilatation, and arteriolar thickening (Prabhakar *et al.* 2007). Then, it has been reported that glomerulosclerosis was highly

increased from 50 weeks of age (Tofovic *et al.* 2000, 2007). With these results, Bilan *et al*. (2011) have suggested that the obese ZSF1 rat may be the best available rat model of human diabetic nephropathy. However, as it is with no intervention, this model is not optimal for the pharmaceutical research requirements, and could be refined to better meet our needs in terms of extent and onset of the metabolic syndrome associated diseases.

2.5.2 Effect of unilateral nephrectomy on renal function of ZSF1 rats

Unilateral nephrectomy (UNx) is a common surgical procedure applied for tumor removal or after accidents. Experimental and human data reported adaptation of the remaining kidney, accompanied by weight increase with a maximal increase of approximately 40% (Drozdzik *et al.* 2003). This functional adaptation involves cell hypertrophy and hyperplasia in human (Fine 1986; Shirley and Walter 1991) as well as in rodents in which hypertrophy is accompanied by pathological changes leading to impaired reduced renal function (Orsic *et al.* 2011).

The fact that renal mass reduction induces glomerular lesions in a metabolic syndrome context is known since many years (Steffes *et al.* 1978; O'Donnell *et al.* 1986). Moreover, it has been demonstrated that nephrectomy exacerbates the remnant renal damage in diabetic rats such as streptozotocin-diabetic rats (Lopes *et al.* 2004) and hyperlipidemic fatty rats (Kasiske *et al.* 1989). As recently pointed out, UNx is an old methodology that is effective to enhance renal alterations under certain context (Katsuda *et al.* 2014).

With this surgical approach we mimicked the method performed in another diabetic rodent model. In diabetic db/db mice, kidney failure was exacerbated and diabetic nephropathy was triggered by renal mass reduction (Ninichuk *et al.* 2007; Tesch and Lim 2011). This was also observed in Sprague-Dawley rats in which UNx deteriorated kidney function (Orsic *et al.* 2011). As previously shown in mice (Gagnon and Ansari 1990), we hypothesized that UNx under dyslipidemia and moderate

hypertension conditions should worsen glomerular injury and albuminuria in the ZSF1 rats. We hypothesized that cardiac function could be deteriorated along with the impairment in kidney function induced by UNx (Sviglerova *et al.* 2010).

PUBLICATION n° 2- SHORT COMMUNICATION

UNILATERAL NEPHRECTOMY ACCELERATES LOSS OF RESIDUAL RENAL FUNCTION IN ZSF1 RATS

Introduction

Comme évoqué précédemment dans la Section 2.1, attendre le développement d'une maladie rénale durant une année chez un modèle animal n'est pas compatible avec les exigences de l'industrie pharmaceutique, en termes de durée. Nous avons donc tenté d'exacerber l'impact du syndrome métabolique sur la dysfonction rénale chez le rat ZSF1, qui pourrait constituer un bon modèle d'étude de la dysfonction rénale dans un contexte de syndrome métabolique, à condition que ces altérations se développent plus rapidement, et de façon plus importante. Pour cela, nous avons utilisé la néphrectomie unilatérale (UNx). Lors d'une première etude, l'UNx a été effectuée chez des rats ZSF1 de 12 semaines, puis nous avons mesuré le taux de filtration glomérulaire à 18 semaines. Dans une seconde étude, l'UNx a également été pratiquée à 12 semaines, et nous avons mesuré différents biomarqueurs de la fonction rénale à 20, 28, 36 et 44 semaines d'âge; l'efficacité du lisinopril en termes de protection rénale a aussi été étudiée. En plus de l'albumine qui est excrétée dans un contexte de maladie rénale, différents biomarqueurs se sont avérés prédictifs des altérations de la structure et de la fonction rénale. Par exemple, une élévation de la cystatine-C est liée à des alterations tubulaires, et l'augmentation de la β2 microglobuline indique une dysfonction glomérulaire (Herrero-Morin *et al.* 2007; Dieterle *et al.* 2010; Fuchs and Hewitt 2011). De plus, Transforming Growth Factor β1 (TGF-β1) a été décrit comme facteur principal responsable de la fibrose rénale (Fan *et al.* 1999; Goumenos *et al.* 2002; Shen *et al.* 2013; Wang *et al.* 2014). Nous avons également mesuré la protéine Monocyte Chemoattractant Protein-1 (MCP-1) dont les niveaux urinaires sont augmentés chez les patients ayant une glomérulopathie (Vianna *et al.* 2013). Notre but étant d'être aussi proche de la maladie humaine que possible du point de vue de son développement, nous avons pensé que pour constituer un bon modèle, le rat ZSF1 devrait montrer une évolution de ces différents biomarqueurs comparable à celle rencontrée chez l'Homme.

Introduction

As stated before in the Section 2.1, more than 50 weeks of development of the renal disease in an animal model is a too long process for the pharmaceutical industry. This constrain pushed many laboratories to investigate new models. We worked on the paths to develop faster and greater renal diseases with features comparable to Human. Based on its characteristics, the ZSF1 rat model could be the suitable model only if its pathological alterations would develop faster and with a greater extent. As a result, and having evaluated evolution of cardiac function with aging in ZSF1 rats, and knowing (according to the literature) that renal function seemed to significantly be impaired around one year of age only, we aimed at exacerbating renal impairment of ZSF1 rat in terms of extent, and duration of onset using UNx. In a first study we performed renal mass reduction by UNx in adult (12 week-old) male ZSF1 rats and evaluated the impact on glomerular filtration rate 6 weeks later, *i.e.* at 18 weeks, compared to age-matched sham-operated ZSF1 rats. In a second study, we performed renal mass reduction by UNx in adult (12 week-old) male ZSF1 rats and evaluated the impact on selected kidney biomarkers at 8, 16, 24 and 32 weeks post UNx (respectively at 20, 28, 36 and 44 weeks of age), and the efficacy of ACEi lisinopril was tested.

Recent development of renal biomarkers has been essential in the stratification of diabetic nephropathy population and monitoring of drugs' efficacy (Fassett *et al.* 2011). Besides albumin (and albumin / creatinine ratio) which is the most abundant protein measured in plasma and excreted by the kidney only in pathological states, several other biomarkers are proposed for qualifying kidney injury. Increase in urinary or plasma β2 Microglobulin (β2M) indicates glomerular dysfunction, and extracellular cysteine proteinase cystatin-C (Cyst-C) speaks for tubule injury (Herrero-Morin *et al.* 2007; Dieterle *et al.* 2010; Fuchs and Hewitt 2011). Mechanistic biomarkers have also been reported, in particular Transforming Growth Factor β1 (TGF-β1) which has been described to be the main inducer of renal fibrogenesis (Fan *et al.* 1999; Goumenos *et al.* 2002; Shen *et al.* 2013; Wang *et al.* 2014). TGF-β1 levels are increased by AngII and its high expression favors apoptosis of podocytes,

tubular epithelial cells and capillary endothelial cells, extra cellular matrix deposition and differentiation of tubular cells into myofibroblasts (Bottinger and Bitzer 2002; Metcalfe 2007; Lee *et al.* 2009). Finally, we measured the Monocyte Chemoattractant Protein-1 (MCP-1) that has been shown increased in patients with a glomerular disease based chronic kidney disease (Vianna *et al.* 2013). Moreover, it was suggested that albuminuria triggers tubular MCP-1 expression with subsequent macrophage infiltration and thus inflammation (Eardley *et al.* 2006). Based on the clinical data collected on kidney biomarkers, we believed that animal models should recapitulate these features to optimally demonstrate drug efficacy and optimize discovery of different classes of drugs.

Experimental study 2 (*JASN*, to be submitted in March)

Unilateral nephrectomy accelerates loss of residual renal function in ZSF1 rats.

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Running Title: UNx and kidney function of ZSF1 rats.

Abstract

Metabolic syndrome, reflected by insulin resistance, atherogenic dyslipidemia, and systemic inflammation leads to type 2 diabetes (T2D) and co-morbidities. Current therapies are not fully effective in preventing loss of kidney function and reducing the cardiovascular risk associated with T2D. One potential reason is the lack of translational animal models of metabolic syndrome that limits drug discovery. We explored the effects of unilateral nephrectomy (UNx) on residual renal function in the male ZSF1 rat, a model of metabolic syndrome. UNx significantly accelerated the onset of kidney structure damage up to 32 weeks post-surgery without changes in glomerular filtration rate or blood pressure. UNx dramatically up regulated biomarkers of tubular and glomerular damage, increased albuminuria and dramatically increased markers of kidney inflammation. Treatment with the Angiotensin Converting Enzyme inhibitor lisinopril prevented renal histological changes and deterioration in renal biomarkers. UNx-ZSF1 rat model presents features that lead to development of kidney failure although a decline in kidney and cardiac function might take longer to develop than without kidney mass reduction. Despite the existence of mouse models of diabetic nephropathy (UNx_db/db, BTBRob/ob or ENOs-/-db/db), no equivalent rat model had been similarly characterized. With these features, UNx-ZSF1 rat represents a translational model to human metabolic syndrome and to kidney failure.

Type 2 Diabetes (T2D) has become a worldwide epidemic (5) requiring more effective and durable therapies. T2D increases the risk of Diabetic Nephropathy (DN) which is the leading cause of Chronic Kidney Disease (CKD) (16) and End-Stage-Renal Disease (ESDR) (6). Metabolic syndrome (MS) (1) often precedes the development of T2D which may allow an earlier intervention point.

In the past decades, research groups have developed rodent models of MS mainly driven by high fat diet (DIO mice and rats) (28), or drug-induced insulin resistance and T2D (7), (11). Relatively limited investigations in these models addressed cardiovascular or renal function loss which are major comorbidities of T2D (29), (21). More recently, the male ZSF1 rat has been more fully characterized as a model of MS (26), in particular for its potential in evaluating drugs to prevent loss of kidney function (27). Although intriguing, the male ZSF1 rat model exhibits a slow rate of kidney function decline (26), with no effect on cardiac function or development of retinopathy (20). Thus, the model as described has limited utility for characterizing potential new drugs for metabolic syndrome co-morbidities.

In this study, we aimed at accelerating the onset of kidney disease of ZSF1 rats (26), (27). We performed renal mass reduction by unilateral nephrectomy (UNx) (15) in adult (12 week-old) male ZSF1 rats and evaluated the impact on kidney function 6 weeks later. This approach mimicked the method performed in diabetic db/db mice, which exacerbated kidney failure and triggered DN (17) (18) (for review, see (25)). UNx significantly reduced GFR from 8.08ml/min/kg bodyweight in sham-operated versus 4.68ml/min/kg UNx-ZSF1 rats ($p<0.001$) (Figure 1A). When expressed in ml/kg/g of kidney, GFR was of 2.01ml/kg/g and 1.83 ml/kg/g in 18 week-old Sham and UNx rats, respectively, suggesting the reduction in glomerular filtration rate (GFR) was solely due to removal of one kidney. UNx had no effect on Mean Arterial Pressure (MAP) up to 24-weeks after renal mass reduction. In UNx-ZSF1 rats, MAP was of approximately 175 mmHg which is comparable to MAP measured in adult naive ZSF1 rats (26).

In absence of GFR and MAP deterioration 6 weeks after UNx, we hypothesized that either UNx has no effect on kidney disease in ZSF1 rats or that more time is required. In order to further explore

these questions, we performed a second study in ZSF1 rats that underwent UNx at the age of 12W and examined kidney structure and function until the age of 44W (Figure 1B). Baseline measurement was set at 20W of age and 8 weeks after UNx (Figure 1B), which corresponds approximately to the timing wherein GFR measurement was unchanged after UNx (Figure 1A). Kidney function was evaluated by quantifying Albuminuria (Alb), Creatinine (Cre) and calculating Alb/Cre ratio (uACR), the biomarkers (BMs) most often used in humans (23). Figure 2A shows that Alb was low at baseline (approx. 250mg/24h) then significantly increased 16-24 and 36-weeks after UNx reaching approximately 800-1000mg/24h) at 44 weeks of age. UNx rapidly increased uACR from approximately 15mg/mg/24h at baseline to 40-54mg/mg/24h, 8-to-24 weeks post UNx (Figure 2B). Deterioration of Alb and uACR (Figure 2) suggests impairment of kidney function. Lisinopril significantly reduced Alb and uACR to levels below baseline values (Figure 2A-B).

Additional urinary BMs were evaluated over 24h at several time points in the study. Cystatin-C (Cyst-C) and β2-microglubulin (β2M) were significantly increased, which indicate deterioration of kidney function (3, 9, 24) and worsening of glomerular filtration (12). TGFβ-1 increase indicated kidney disease progression and loss of tubular function (14). Increased Monocyte Chemoattractant Protein-1 (MCP-1) suggested increase in inflammation and macrophage infiltration (8). UNx dramatically increased urinary levels of these four BMs in ZSF1 rats (Figure 2C-D) with different temporal pattern: increase in TGFβ -1 and MCP-1 occurred earlier (Figure 2D) compared to Cyst-C and β2M (Figure 2C). Lisinopril was able to significantly reduced UNx-induced increase in BMs (Figure 2C-D) to levels equivalent to baseline (Alb, Cyst-C and β2M) or even below baseline (uACR, TGF β-1 and MCP-1) (Figure 2).

Removal of one kidney dramatically altered of the histology of the remaining kidney. Glomeruli of ZSF1 were mainly healthy at baseline since grade 0 glomerulosclerosis (GS) was observed in more than 80% of the glomeruli (Figure 3A). GS in ZSF1 rats progressively worsened after UNx, as seen by the increase in grades 1-4 and reduction of grade 0 (Figure 3A). Lisinopril efficiently prevented development of GS after 1-or-2 weeks of treatment. UNx deteriorated kidney sum score to a score

above 20 (Figure 3B). UNx worsened GS and thickening of basement membrane (Figure 3C) and induced inflammation as shown by reduced macrophage infiltration (increase in CD68 staining) (Figure 3D). Treatment with Lisinopril strongly reduced kidney structural damage induced by UNx (Figure 3A-B). UNx had no effects in other organs (eyes, liver, heart) of ZSF1 rats indicating the absence of retinopathy or cardiac changes as a consequence of UNx until the age of 44W (data not shown).

In summary, we aimed at exploring the impact of renal mass reduction (15) as a way to exacerbate the onset of CKD in the male ZSF1 rat model of MS. Despite the existence of mouse models of DN such as UNx_db/db, BTBRob/ob or ENOs-/-db/db (2, 13), (17), (25), (31), no rat model of T2D or MS has been similarly characterized. Moreover, the use of very small rodents, like mice, favors the estimated GFR (eGFR) instead of measured GFR or Cre Clearance which are too imprecise measurements for determining accurately kidney function (25), (22).

In humans, loss of kidney function is witnessed by drop in creatinine clearance which is usually associated with increase in Alb and proteinuria. Impact on kidney structure and other BMs are often not evaluated. It was suggested that other kidney BMs than Alb could be more sensitive to early renal damage and might be significantly deteriorated prior to rise of Alb (10). In this work, we reported the combined of effect of UNx on truly measured GFR (not eGFR), Alb and a panel of additional BMs (describing function of different kidney structures) and kidney structure examined by semi-quantitative histology and staining. This triangular assessment showed that UNx rapidly and significantly deteriorated BMs, kidney structures and increased inflammation, lymphocytes and macrophages infiltration. GFR appeared worsened by UNx when expressed as ml/min/kg BW but not when adjusted to one left kidney and expressed as ml/min/g kidney weight, demonstrating that worsening of kidney BMs occur prior to GFR decline. Adaptation of kidney function right after UNx is rendered possible due to hyperfiltration of the left kidney (4) (30). The worsening of kidney BMs translates the ongoing changes that announce the progressive loss of kidney function.

With these data, we propose the modified animal model of MS (UNx-ZSF1) as a tool mimicking steps and features of human kidney disease. The triangular determination of BMs, GFR and histology examination is required to define kidney function status. In this study, we showed that UNx-ZSF1 rats was sensitive to lisinopril (Angiotensin Converting Enzyme inhibitor, ACEi) administered at a relevant therapeutic dose of 30mg/kg/d. Lisinopril significantly reduced MAP by 29% (from 175 down to 124 mmHg, $p<0.001$) and 31% (from 173 down to 119 mmHg, $p<0.001$) compared to vehicle, after 8 weeks and 16 weeks of treatment, respectively. While UNx worsened DN progression of ZSF1 rats, lisinopril improved BMs to a similar or greater extent compared to baseline, and protected kidney structures.

Our data showed that UNx-ZSF1 rat model faster develops main features of human CKD. Newly characterized UNx-ZSF1 rats could represent a tool model to evaluate efficacy of new drugs protecting kidney from loss of function.

Conflict of Interest

None

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Concise methods

Studies were performed under animal research protocol (ARP) approved by the Swiss Cantonal Veterinary Office Basel-Stadt and in accordance with guidelines of the Association for the Assessment and Accreditation of Laboratory and Animal Care (AAALAC # Unit Number: 001057). Male ZSF1 rats [(OB) Gmi Crl-fa/fa] were ordered at the age 10W from Charles River U.S.A. and fed a special diet equivalent to PURINA PMI 5008 (Ssniff, Soest, Germany). Rats were group-housed at approximately 21°C and 55–65% relative humidity and had ad libitum access to food and water. Light cycle was from 6 a.m. to 6 p.m.

At the age of 12W, rats underwent unilateral nephrectomy. Rats were treated with buprenorphine (0.05mg/kg, subcutaneous) one hour prior to surgery to minimize pain. Rats were anesthetized with isoflurane/O2. Rat was placed upon a heated operating surface to maintain body temperature at 37°C. The left kidney flank was accessed via a small lateral flank incision and exposed allowing the access to the renal pedicle. The vascular pedicle was occluded with a titanium clip ligature medium (Braun Aesculap) followed by kidney removal. The flank incision was sutured and the skin was closed with atraumatic clips (3M- DS 25 Precise disposable skin stapler). Each rat received buprenorphine (0.05mg/kg/day, subcutaneous) for the two days following surgery in order to minimize pain. Each animal was placed back in its cage and single housed for at least one week to allow full recovery. A group of sham-operated ZSF1 rats (that underwent anesthesia, left flank opening, then suture) and a group of age-matched UNx-ZSF1 rats were constituted. This small cohort of ZSF1 rats was used to determine GFR, 6 weeks after surgery, i.e. at the age of 18 weeks (cf. data in Figure 1A).

Eight weeks after UNx was performed, ZSF1 rats (at 20 weeks of age) were randomized according to body weight and urine biomarkers for kidney function (Albuminuria, proteinuria and Cystatin-C). Four Cohorts of rats were constituted and sacrificed at baseline (8 weeks after UNx), and 16w-, 24W- and 32w post Unx. Groups of n=6-8 were constituted. Rats were either treated with vehicle or with

Lisinopril (30mg/kg/d) that was mixed in food (food admix conditions) for 8- and 16-weeks, respectively. A last group of UNx_ZSF1 rats was kept untreated until the age of 44w, i.e. 32 weeks after UNx was performed. This group of UNx-ZSF1 rats was used to evaluate the kidney disease progression after renal mass reduction.

Systemic systolic and diastolic arterial blood pressure (SAP, DAP) measurements were performed non-invasively at the end of each the treatment's period i.e. after completion of 8- and 16 weeks treatment with Lisinopril. Blood pressures were measured in conscious animals with a computerized tail-cuff system, based on the Volume Pressure Recording technology (Model CODA; Kent Scientific Corporation, USA). SAP and DAP were measured 2 hours after acclimation in a dedicated room heated at 28°C.

Urine was collected once at baseline, then at the end of each treatment period. Rats were placed into metabolic cages with ad libitum access to water and food. The urine samples collected for 24h were maintained at +4°C. Urine samples were centrifuged at 800 RPM for 10 minutes and supernatant was taken for analysis.

Blood was collected under 4h fasting conditions (in the morning) in EDTA-coated tubes at necropsy after decapitation under isoflurane anesthesia. Blood samples were centrifuged (at +4°C, 10000 RPM, for 15 minutes), then plasmas were frozen (-20°C) and stored for bio-analytical analysis.

Commercially available rat enzyme kits for quantifying urinary Albumin (Alpco, 41-ALBRT-E01), monocyte chemoattractant protein-1 (MCP-1, R&D Systems, SMJE00) and transforming growth factor beta-1 (TGF-β1, BioLegend, 436706) and β2-microglobulin (β2M), Cystatin-C (Cyst-C) (Millipore) were used according to the manufacturer's instructions. Concentrations of all urinary biomarkers were normalized to urine volume.

Measurement of GFR was performed in 18w-old ZSF1 rats using the Fluorescein isothiocyanate (FITC)-Inulin clearance method (19). Two groups of rats were evaluated: naïve (sham) or those that underwent renal mass reduction by UNx at the age of 12w. GFR was determined after FITC-inulin (Sigma – Aldrich, St. Louis, MO, USA; cat no F3272, 40mg/mL in 0.9% NaCl) was injected (1mL/kg,

intra venous). Blood samples (via tail nick, volume ~100µL each) were collected in EDTA coated tubes (Microvette 300µL, SARSTEDT, D-51588 Nümbrecht, Germany) at +0.5, 3, 7, 10, 15, 20, 40 and 60 min after inulin injection. The samples were kept on ice. Plasma, generated as described above, was stored in 96 well plates at -20°C before measurement of inulin. GFR was calculated for each individual rat then averaged for each treatment group.

Measurement of FITC Inulin in plasma: Because pH significantly affects FITC fluorescence, plasma samples were buffered to pH 7.4 with 500mM HEPES. To 40µL of HEPES buffer in each well of a 96 well plate (Costar, #3694), 10µL plasma were added and mixed. Fluorescence was determined using a microplate reader (Infinite M200 Pro, Tecan) with 485nm excitation and read at 538nm emission.

Kidney, heart and liver were harvested at the end of the study and weighed. Heart and liver were then immediately immersed in 10% neutral buffered formalin for 24h. Only histological data on kidney structure is shown. Kidney was divided in two pieces cut longitudinally that were placed in 10% neutral buffered formalin for maximum 24h. After rinsing, harvested tissues were embedded in paraffin blocks and slices of 4µm were prepared. Sections were stained with standard hematoxylin eosin (HE; all tissues) and Periodic acid-Schiff reaction (PAS; kidney and eyes). For the detection of macrophages in the kidney, de-paraffinized sections were stained with the Ventana DAB Map TM kit using the avidin biotin peroxidase complex method involving a biotinylated secondary antibody of donkey-anti-mouse on a Ventana Discovery XT immunostainer (Ventana medical System, Tucson, Arizona). The primary antibody used was a pre-diluted (1:100) monoclonal mouse-anti-CD68 antibody (Abcam, ab31630).

A scoring system for kidney structures changes including glomerular vacuolation, thickening of basement membranes, interstitial inflammation, interstitial fibrosis, tubular dilation, proteinaceous casts, was established as follows: grade 0 (no change), grade 1 (minimal; ≤ 10% of a structure affected), grade 2 (slight; between 10 and 20% of a structure affected), grade 3 (moderate; between 20 and 50% of a structure affected), grade 4 (marked; between 50 and 75% of a structure affected), and grade 5 (severe; up to 100% of a structure affected).

For GS, 50 glomeruli per kidney were examined and each scored into grade 0 (no glomerulosclerosis), grade 1 (≤ 25% of glomerulus affected), grade 2 (≤ 50% of glomerulus affected), grade 3 (≤ 75% of glomerulus affected), and grade 4 (up to 100% of glomerulus affected). For the calculated GS score for each kidney, the sum of each glomerulus multiplied with the score was calculated, and the obtained sum was graded as follows: grade 1 (sum ≤ 25), grade 2 (sum between 26 and 50), grade 3 (sum between 51 and 75) grade 4 (sum between 76 and 100) grade 5 (sum > 100).

Statistical analysis

Data are shown as mean ± SEM. Group size was of 6-8 ZSF1 rats. Statistical analysis was performed using statistical tools in Excel (F-Test followed by T-Test). Each study group consisted of vehicle and lisinopril-treated rats except for the oldest ZSF1 rats (44w-old at the end of the study) that were not treated with lisinopril. Statistical significance was * $P<0.05$, ** $p<0.01$ or *** $p<0.001$ versus vehicle.

References

1. Alberti G, Zimmet P, Kaufman F, Tajima N, Silink M, Arslanian S, Womg G, Bennett P, Shaw J, and Caprio S. METABOLIC SYNDROME - in children and adolescents. IDF communication, 2007.

2. Alpers CE and Hudkins KL. Mouse models of diabetic nephropathy. Curr Opin Nephrol Hypertens 20: 278-284, 2011.

3. Alter ML, Kretschmer A, Von Websky K, Tsuprykov O, Reichetzeder C, Simon A, Stasch JP, and Hocher B. Early urinary and plasma biomarkers for experimental diabetic nephropathy. Clinical laboratory 58: 659-671, 2012.

4. Brenner BM. Nephron adaptation to renal injury or ablation. The American journal of physiology, 249: F324-337, 1985.

5. Danaei G, Finucane MM, Lu Y, Singh GM, Cowan MJ, Paciorek CJ, Lin JK, Farzadfar F, Khang Y-H, Stevens GA, Rao M, Ali MK, Riley LM, Robinson CA, and Ezzati M. National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2•7 million participants. The Lancet 378: 31-40, 2011.

6. De Boer IH. CHronic kidney disease—a challenge for all ages. JAMA 308: 2401-2402, 2012.

7. De Nicola AF FO, Del Castillo EJ ,Foglia VG. The Influence of Streptozotocin Diabetes on Adrenal Function in Male Rats. Horm Metab Res 8: 388-392, 1976.

8. Deshmane SL, Kremlev S, Amini S, and Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res 29: 313-326, 2009.

9. Dharnidharka VR, Kwon C, and Stevens G. Serum cystatin C is superior to serum creatinine as a marker of kidney function: A meta-analysis. Am-J-Kidney-Dis 40: 221-226, 2002.

10. Fassett RG, Venuthurupalli SK, Gobe GC, Coombes JS, Cooper MA, and Hoy WE. Biomarkers in chronic kidney disease: a review. Kidney International 80: 806-821, 2011.

11. G T, S R-M, and A N. Long-term changes in the diabetic state induced by different doses of streptozotocin in rats. British Journal of Experimental Pathology 64: 117-123, 1983.

12. Herrero-Morin JD, Malaga S, Fernandez N, Rey C, Dieguez MA, Solis G, Concha A, and Medina A. Cystatin C and beta2-microglobulin: markers of glomerular filtration in critically ill children. Crit Care 11, 2007.

13. Hudkins KL, Pichaiwong W, Wietecha T, Kowalewska J, Banas MC, Spencer MW, Muhlfeld A, Koelling M, Pippin JW, Shankland SJ, Askari B, Rabaglia ME, Keller MP, Attie AD, and Alpers CE.

BTBR Ob/Ob mutant mice model progressive diabetic nephropathy. J Am Soc Nephrol 21: 1533-1542, 2010.

14. Lee SB, Kanasaki K, and Kalluri R. Circulating TGF-beta1 as a reliable biomarker for chronic kidney disease progression in the African-American population. Kidney International 76: 10-12, 2009.

15. Liu C, Chen Y, Kang Y, Ni Z, Xiu H, Guan J, and Liu K. Glucocorticoids Improve Renal Responsiveness to Atrial Natriuretic Peptide by Up-Regulating Natriuretic Peptide Receptor-A Expression in the Renal Inner Medullary Collecting Duct in Decompensated Heart Failure. Journal of Pharmacology and Experimental Therapeutics 339: 203-209, 2011.

16. Martini S, Nair V, Keller BJ, Eichinger F, Hawkins JJ, Randolph A, Böger CA, Gadegbeku CA, Fox CS, Cohen CD, Kretzler M, Bank tERc, Cohort C-P, and Consortium C. Integrative Biology Identifies Shared Transcriptional Networks in CKD. Journal of the American Society of Nephrology 25: 2559-2572, 2014.

17. Ninichuk V, Kulkarni O, Clauss S, and Anders H. Tubular atrophy, interstitial fibrosis, and inflammation in type 2 diabetic db/db mice. An accelerated model of advanced diabetic nephropathy. European journal of medical research 12: 351, 2007.

18. Orsic V, Mihalj M, Mogus M, Mihalj H, Jakic M, Heffer-Lauc M, Marjanovic K, and Zibar L. Impaired kidney function in rats six months after unilateral nephrectomy - an old story, a new perspective. Med Glas (Zenica) 8: 185-191, 2011.

19. Qi Z and Breyer MD. Measurement of glomerular filtration rate in conscious mice. Methods Mol Biol 466: 61-72, 2009.

20. Riboulet W, Hainaut E, Verry P, Lambotte J, Jacobsen B, Lartaud I, Bénardeau A, and Conde-Knape K. Longitudinal assessment of heart function of ZSF1 rats by echocardiography. Journal of Experimental and Applied Animal Science 1: 125-139, 2013.

21. Shao N, Kuang HY, Wang N, Gao XY, Hao M, Zou W, and Yin HQ. Relationship between Oxidant/Antioxidant Markers and Severity of Microalbuminuria in the Early Stage of Nephropathy in Type 2 Diabetic Patients. Journal of Diabetes Research 2013: 6, 2013.

22. Sharma K, McCue P, and Dunn SR. Diabetic kidney disease in the db/db mouse. Am-J-Physiol-Renal-Physiol 284: F1138-1144, 2003.

23. Simonson MS, Tiktin M, Debanne SM, Rahman M, Berger B, Hricik D, and Ismail-Beigi F. The renal transcriptome of db/db mice identifies putative urinary biomarker proteins in patients with type 2 diabetes: a pilot study. Am-J-Physiol-Renal-Physiol 302: F820-829, 2012.

24. Song S, Meyer M, Türk TR, Wilde B, Feldkamp T, Assert R, Wu K, Kribben A, and Witzke O. Serum cystatin C in mouse models: a reliable and precise marker for renal function and superior to serum creatinine. Nephrology Dialysis Transplantation 24: 1157-1161, 2009.

25. Tesch GH and Lim AK. Recent insights into diabetic renal injury from the db/db mouse model of type 2 diabetic nephropathy. Am-J-Physiol-Renal-Physiol 300: F301-310, 2011.

26. Tofovic SP and Jackson EK. Rat Models of the Metabolic Syndrome. In: Renal Disease -Methods in Molecular Medicine-, 2003, p. 29-46.

27. Tofovic SP KH, Kost CK Jr, Bastacky S. Renal function and structure in diabetic, hypertensive, obese ZDFxSHHF-hybrid rats. Ren Fail 22: 387-406, 2000.

28. Tschöp M and Heiman ML. Rodent obesity models: an overview. Exp-Clin-Endocrinol-Diabetes 109: 307-319, 2001.

29. Wilson PWF, D'Agostino RB, Parise H, Sullivan L, and Meigs JB. Metabolic Syndrome as a Precursor of Cardiovascular Disease and Type 2 Diabetes Mellitus. Circulation 112: 3066-3072, 2005.

30. Yoshida Y, Fogo A, and Ichikawa I. Glomerular hemodynamic changes vs. hypertrophy in experimental glomerular sclerosis. Kidney International 35: 654-660, 1989.

31. Yuen DA, Stead BE, Zhang Y, White KE, Kabir MG, Thai K, Advani SL, Connelly KA, Takano T, Zhu L, Cox AJ, Kelly DJ, Gibson IW, Takahashi T, Harris RC, and Advani A. eNOS deficiency predisposes podocytes to injury in diabetes. J Am Soc Nephrol 23: 1810-1823, 2012.

Figures

Figure 1: **GFR measured in 18w-old ZSF1 rats submitted or not to unilateral Nephrectomy (A). GFR was expressed in relation to body weight or kidney weight (right). Values are mean ± SEM. N=6-8 per group. F test for determining variance, then followed by T test, ***P < 0.001 vs. vehicle. Study design implemented in 4 cohorts of UNx-ZSF1 rats (B).**

Figure 2: **Albuminuria (Alb) (A), Alb/Cre ratio (ACR) (B), Cyst-C, β2M (C) and TGF β-1, MCP-1 (D) measured in UNx-ZSF1 rats under vehicle, or Lisinopril (30 mg/kg) treatment. N=6-8 per group. Data expressed as mean +/- SEM; N=6-8 per group; F test for determining variance followed by T test, *p<0.05, **p<0.01, ***p<0.001 vs. vehicle. Vehicle Baseline corresponds to untreated UNx-ZSF1rats. Black bars correspond to UNx-ZSF1 rats that were kept untreated during the whole study duration, i.e. until 32 weeks post UNx.**

Figure 3: **Histopathology of kidney of ZSF1 rats along disease progression after UNx. Values are mean ± maximum and minimum findings. N=6-8 per group.**

Kidney mean differential score glomerulosclerosis (GS) evaluated in ZSF1 rats (**A**). Kidney Score summarizing separate kidney findings in ZSF1 rat **(B)**. The kidney structures considered in the kidney score are: GS, Glomerular vacuolation, thickening of basement membrane, interstitial fibrosis, interstitial inflammation, tubular dilation and proteinaceous cast. Histopathology of kidneys of UNx ZSF1 rats **(C)**. Infiltration of CD68 positive cells (macrophages) in kidneys of ZSF1 rats (**D**). ZSF1 rats were 20w-old and examined 7 weeks after UNx (**a**), ZSF1 rats were 28w-old and examined 16 weeks after UNx (**b**), ZSF1 rats were 36w-old and examined 24 weeks after UNx (**c**) or ZSF1 rats were 44w-old and examined 32 weeks after UNx (**d**). Immunohistochemistry with an antibody directed against CD68, DAB stain. Basement membrane thickening (arrow) and glomerulosclerosis (star); PAS stain.

Figure 4: **Histopathology of kidneys of 36w-old ZSF1 rats, 24 after UNx, under vehicle (a) or Lisinopril (b) treatment. Basement membrane thickening (arrow) and glomerulosclerosis (star); PAS stain. Infiltration of CD68 positive cells (macrophages) in kidneys of 36-week-old ZSF1 rats, 24w after UNx, treated with Vehicle (c) or Lisinopril (d). Immunohistochemistry with an antibody directed against CD68, DAB stain.**

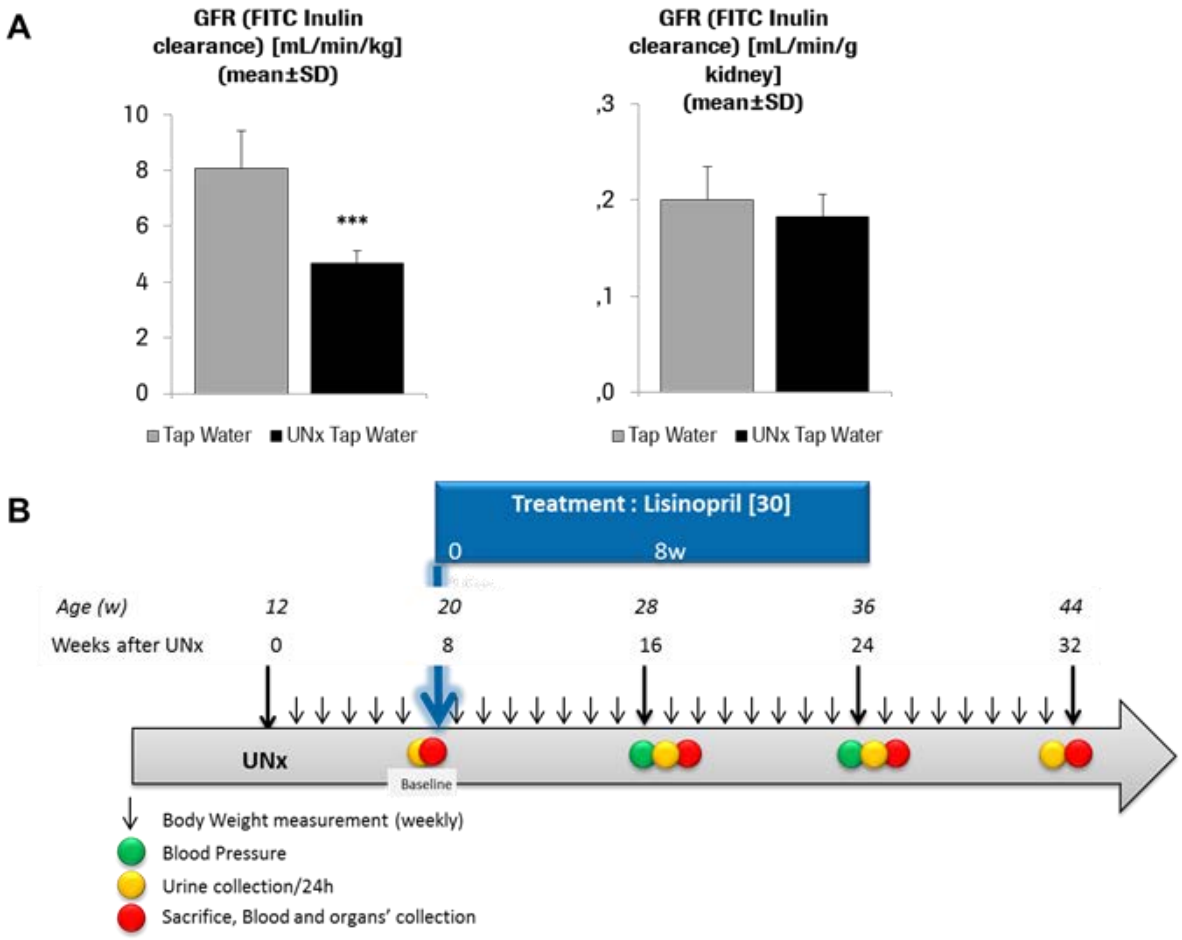

A
GFR (FITC Inulin clearance) [mL/min/kg] (mean±SD)
10
8
6
4
2
0
***
Tap Water
UNx Tap Water
GFR (FITC Inulin clearance) [mL/min/g kidney] (mean±SD)
,3
,2
,1
,0
Tap Water
UNx Tap Water
B
Treatment : Lisinopril [30]
0
8w
Age (w)
12
20
28
36
44
Weeks after UNx
0
8
16
24
32
UNx
Baseline
Body Weight measurement (weekly)
Blood Pressure
Urine collection/24h
Sacrifice, Blood and organs' collection

Figure 1

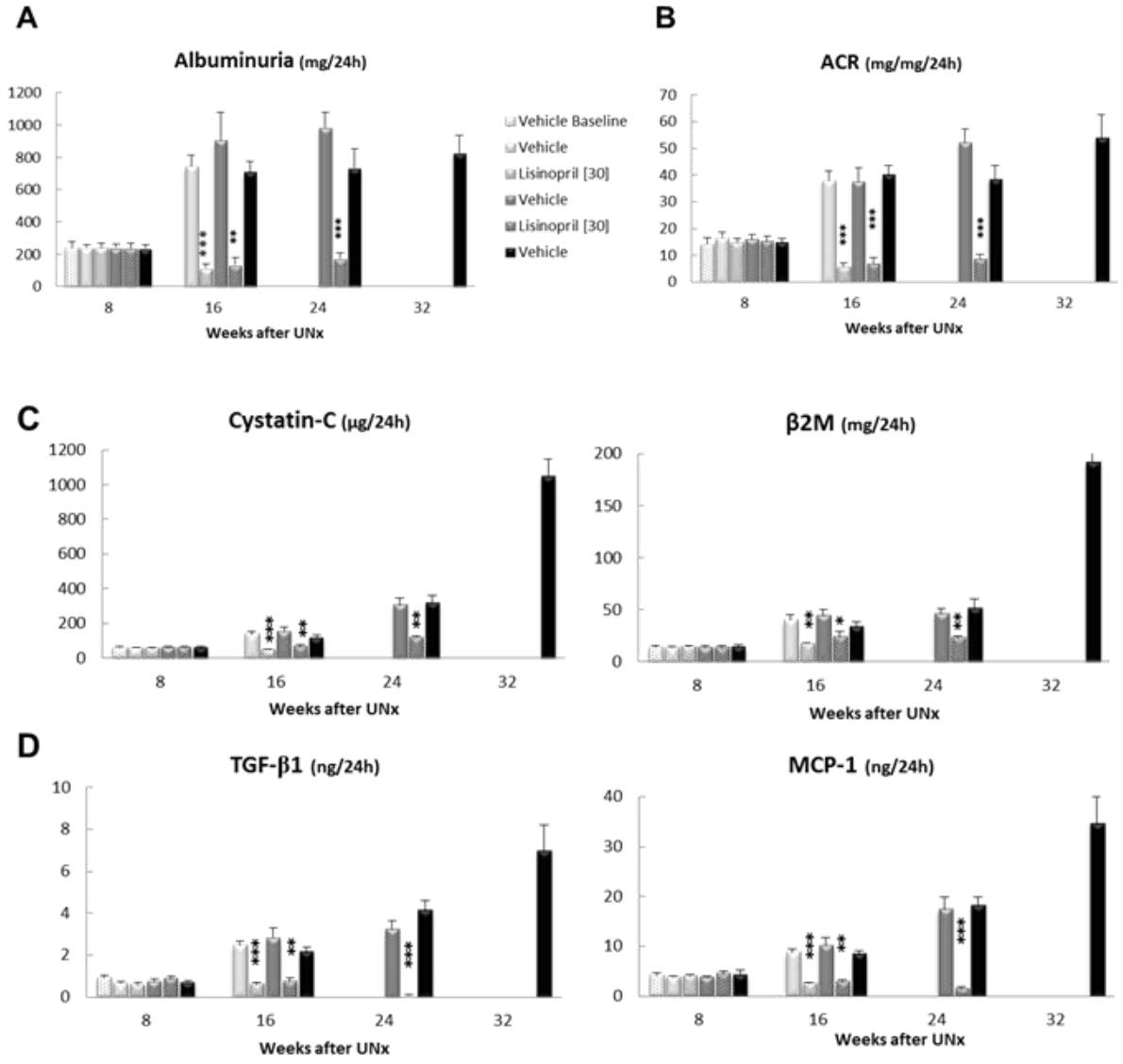
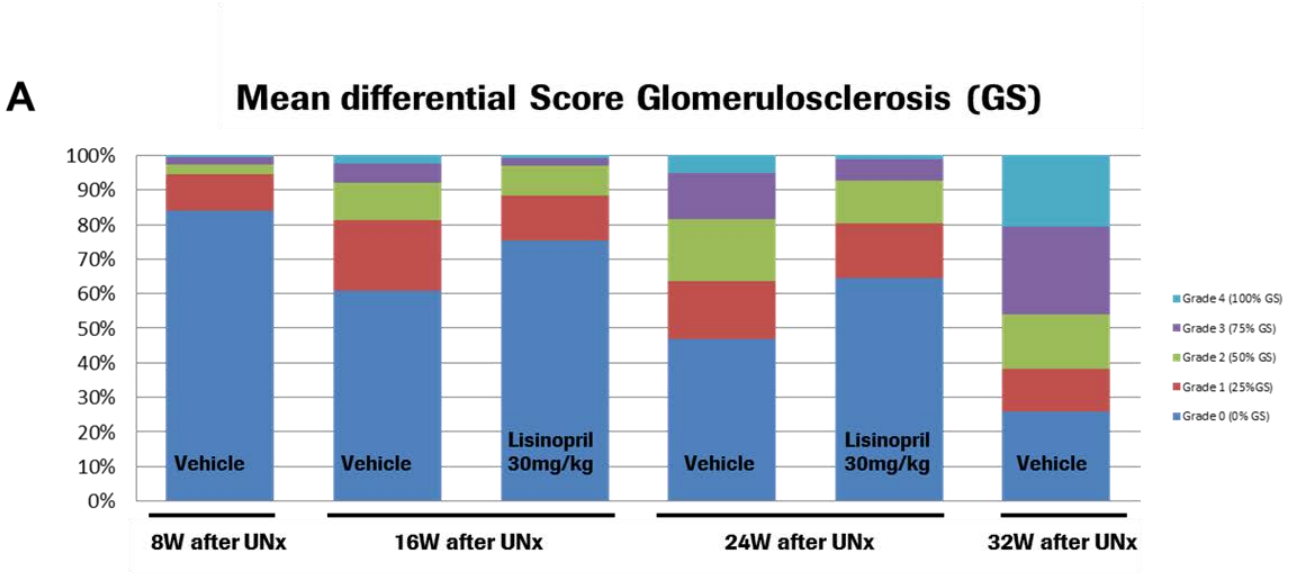
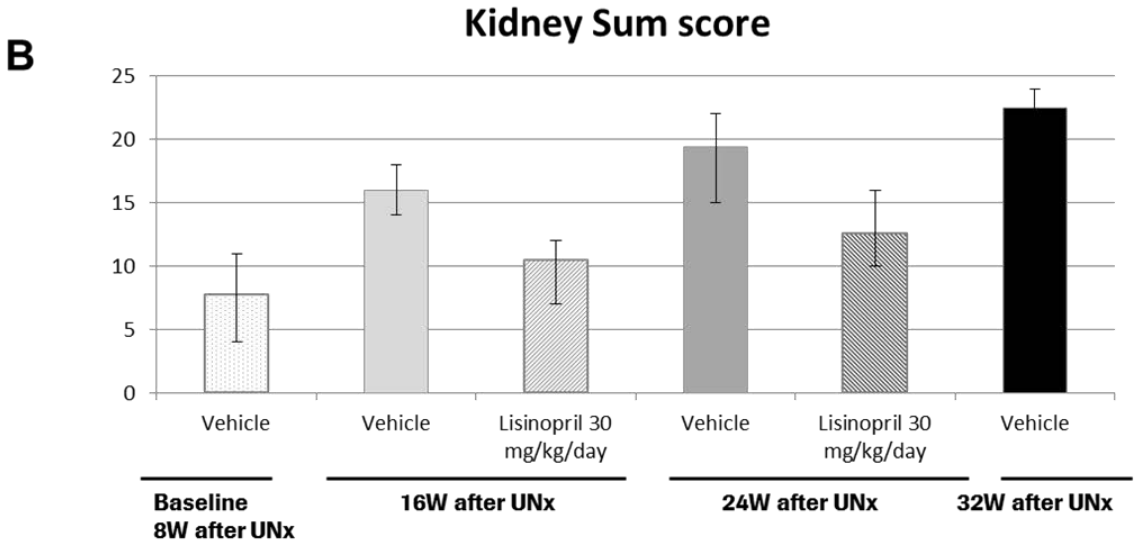
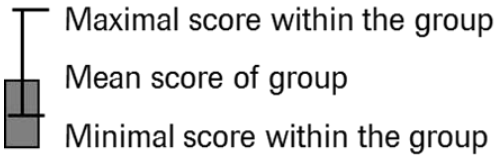

A
Albuminuria (mg/24h)
Vehicle Baseline
Vehicle
Lisinopril [30]
Vehicle
Lisinopril [30]
Vehicle
Weeks after UNx
B
ACR (mg/mg/24h)
Weeks after UNx
C
Cystatin-C (µg/24h)
Weeks after UNx
β2M (mg/24h)
Weeks after UNx
D
TGF-β1 (ng/24h)
Weeks after UNx
MCP-1 (ng/24h)
Weeks after UNx

Figure 2


A
Mean differential Score Glomerulosclerosis (GS)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Vehicle
Vehicle
Lisinopril 30mg/kg
Vehicle
Lisinopril 30mg/kg
Vehicle
8W after UNx
16W after UNx
24W after UNx
32W after UNx
Grade 4 (100% GS)
Grade 3 (75% GS)
Grade 2 (50% GS)
Grade 1 (25%GS)
Grade 0 (0% GS)


B
Kidney Sum score
25
20
15
10
5
0
Vehicle
Vehicle
Lisinopril 30 mg/kg/day
Vehicle
Lisinopril 30 mg/kg/day
Vehicle
Baseline 8W after UNx
16W after UNx
24W after UNx
32W after UNx


Maximal score within the group
Mean score of group
Minimal score within the group

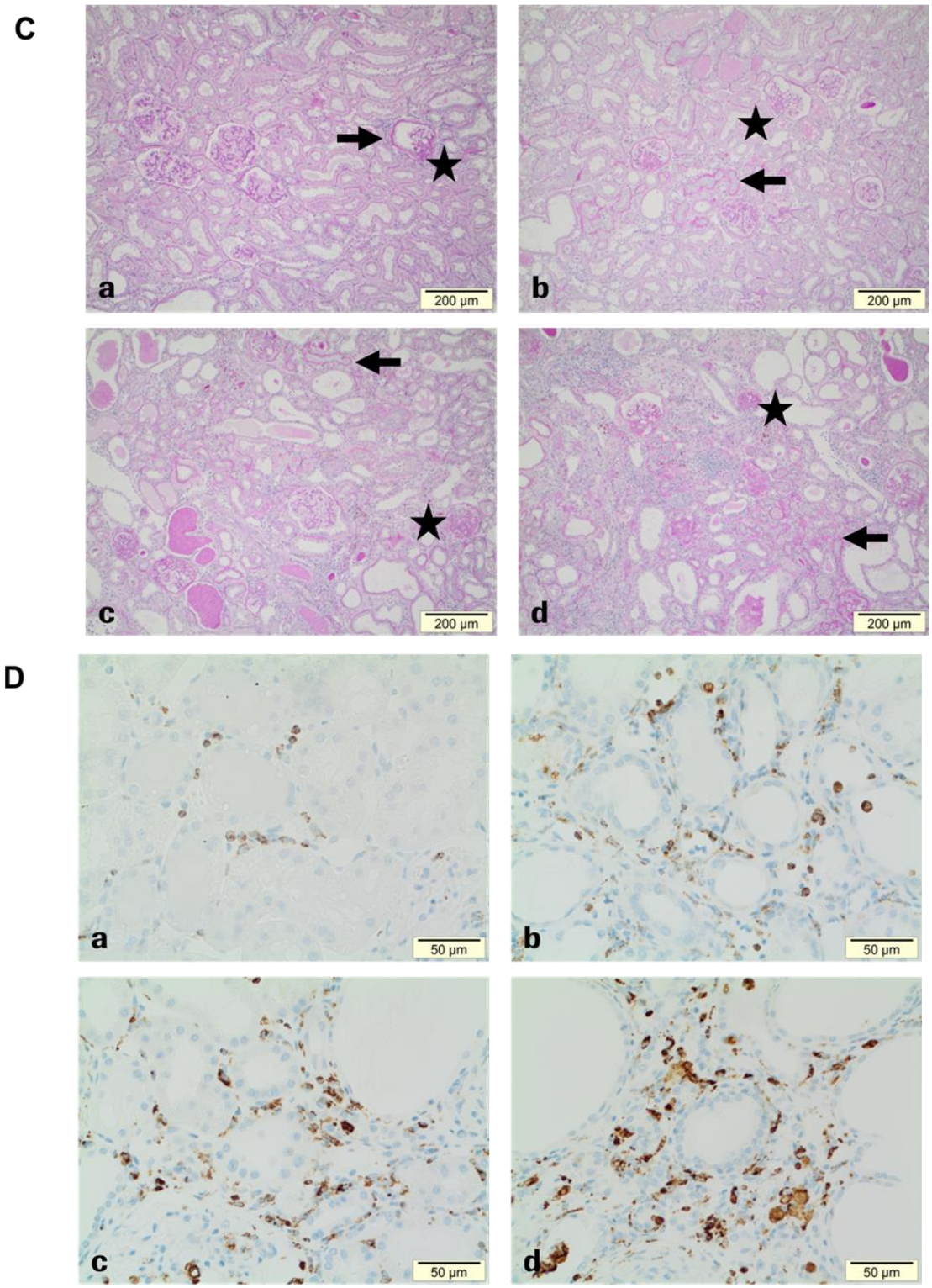


Figure 3

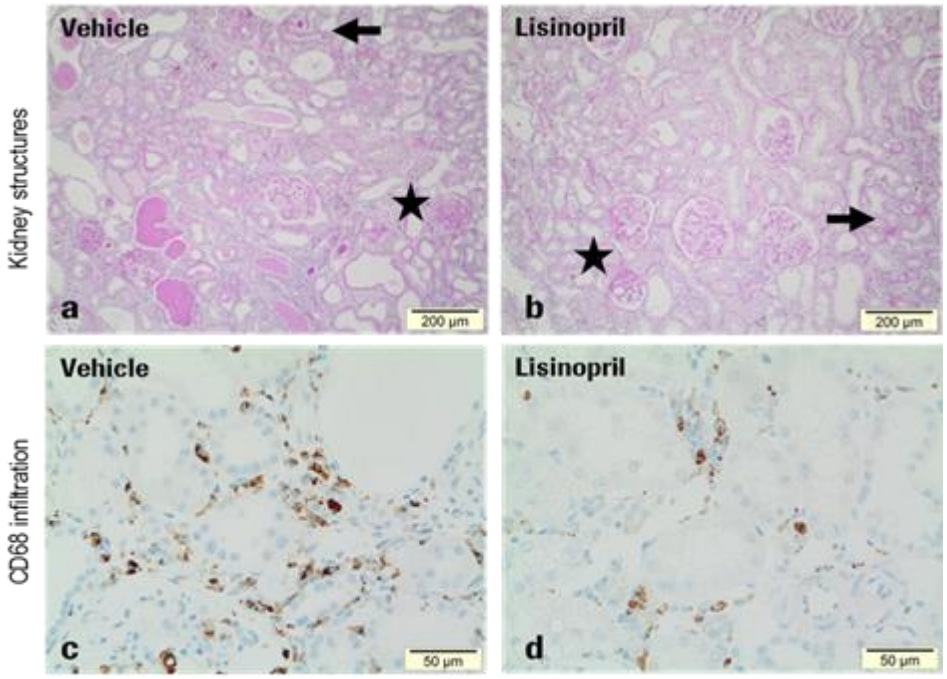

Vehicle
Lisinopril
Kidney structures
a
200 µm
b
200 µm
Vehicle
Lisinopril
CD68 infiltration
c
50 µm
d
50 µm

Figure 4

Discussion - Conclusion

Pour résumer, nous avons voulu, par l'utilisation de l'UNx, engendrer une dégradation plus rapide et plus importante de la function rénale chez le rat ZSF1, dans un contexte de syndrome métabolique. La première étude a montré que le taux de filtration glomérulaire n'était pas impacté 6 semaines après l'UNx, la seconde que la réduction de la masse rénale induisait une dégradation rapide et significative des biomarqueurs mesurés et de la structure rénale, et une aggravation de la fonction associée. Nous avons montré que l'UNx permettait une déterioration rénale plus rapide, avec des caractéristiques communes avec la situation humaine, dans un context de syndrome métabolique. Ainsi le rat ZSF1 couplé à l'UNx pourrait permettre de tester l'efficacité de composés sur la protection rénale. Dans une étude pilote, nous avions mesuré l'impact de l'UNx sur la fonction cardiaque qui s'était montré nul (voir annexe 1). Cependant, nous avons décidé de tester l'impact de l'anti-oxydant bardoxolone sur la fonction rénale de ces ZSF1-UNx, désormais altérée en un temps plus raisonnable.

Discussion - Conclusion

In summary, we aimed at exacerbating the onset of chronic kidney disease in the male ZSF1 rat model of metabolic syndrome. In this work, we showed in study 1 that 6 weeks post UNx was not a long enough duration to alter GFR. The second study showed that UNx rapidly and significantly deteriorated biomarkers, kidney structure and increased inflammation, lymphocytes and macrophages infiltration.

We showed that UNx-ZSF1 rat model was sensitive to lisinopril (ACEi) administered at a relevant therapeutic dose of 30mg/kg/d. Our data showed that UNx-ZSF1 rat model faster developed the main features of human chronic kidney disease in a context of metabolic syndrome. Newly characterized UNx-ZSF1 rats could represent a tool model to evaluate efficacy of new drugs protecting kidney from loss of function.

Regarding the cardiac function, in a previous pilot study, we compared the systolic and diastolic functions in UNx animals *vs*. sham operated animals, and we concluded that neither systolic function (*i.e.* ejection fraction) nor diastolic function (*i.e*. E/A ratio) were impacted by UNx (See annex 1). Although cardiac function was not altered with UNx, we decided to pursue with the UNx-ZSF1 rat model to test this time a compound that was not related with the RAAS system, as in the previous Short Communication, *i.e.* the antioxidant bardoxolone, on kidney function.

2.5.3 Effect of the antioxidant bardoxolone on kidney function of unilateral nephrectomized ZSF1 rats

PUBLICATION n° 3

BARDOXOLONE METHYL DECREASED BODY WEIGHT, IMPROVED KIDNEY FUNCTION IN MALE ZSF1 RATS ASSOCIATED WITH SPECIFIC GENE CHANGES AND SIGNS OF TOXICITY

Introduction

Les traitements actuels pour prévenir ou traiter la néphropathie chronique ne donnent pas entière satisfaction. L'état pro-inflammatoire caractérisant le syndrome métabolique est souvent retrouvé chez les patients développant une néphropathie chronique (Nguyen *et al.* 2006; Shaker and Sadik 2013; You *et al.* 2013), ainsi que chez les animaux (Chow *et al.* 2005), en plus de l'hyperglycémie, d'un stress oxydant et de l'hypertension, caractérisant le syndrome métabolique (Heloisa *et al.* 2005; Cachofeiro *et al.* 2008). Depuis de nombreuses années, les composés antihypertenseurs comme les ACEi ou les ARBs et les diurétiques sont les traitements les plus préconisés pour traiter ces néphropathies. D'autres agents aux propriétés anti-oxydantes ou anti-inflammatoires ont plus récemment été testés et ont montré un intérêt quant à la protection rénale. Parmi les composés anti-oxydants, le bardoxolone a montré des résultats encourageants sur la protection rénale chez des patients présentant une néphropathie chronique (Pergola *et al.* 2011). Cependant, un risque accru de développer une maladie cardiovasculaire telle qu'un infarctus du myocarde, ou une insuffisance cardiaque, a été imputé à l'utilisation du bardoxolone, ce qui a conduit à l'arrêt prématuré d'une seconde étude clinique menée plus récemment (de Zeeuw *et al.* 2013). Nous avons choisi d'évaluer les effets du bardoxolone sur la protection rénale, et de les comparer à ceux induits par le lisinopril. Pour cela, l'UNx a été effectuée à 12 semaines, les traitements ont été donnés dix semaines plus tard (soit à 22 semaines) associés à la nourriture pendant 8 semaines. La fonction rénale a été évaluée par la mesure plasmatique et urinaire de biomarqueurs listés auparavant, ainsi que par celle de la protéine membranaire Kidney Injury Molecule (KIM-1), exprimée lors de lésions rénales par les cellules épithéliales tubulaires proximales chez l'Homme et chez la souris (Han *et al.* 2002; Lock 2010; Fuchs and Hewitt 2011; Kirk 2013; Luk *et al.* 2013). A la fin des 8 semaines de traitement, soit

à 30 semaines d'âge, la fonction rénale a été déterminée plus précisément par la mesure du taux de filtration glomérulaire.

Introduction

The existing treatments for preventing or curing chronic kidney disease progression are not fully satisfactory. The underlying pro-inflammatory state attributed to obesity often found in chronic kidney disease patients, has been implicated in chronic kidney disease development both in animal models (Chow *et al.* 2005) and in patients (Nguyen *et al.* 2006; Shaker and Sadik 2013; You *et al.* 2013) on top of hyperglycemia, oxidative stress and hypertension, that all together constitute the metabolic syndrome (Heloisa *et al.* 2005; Cachofeiro *et al.* 2008). Since decades, the most frequently used standards of care are diuretics, anti-hypertensive drugs (ACEi, ARBs). Since few years, tentatives to develop alternative treatments to standard of care have been investigated such as anti-inflammatory and anti-oxidant. Those new medical entities have been shown to protect kidney function from ischemia reperfusion insults. They looked as possible promising treatment for chronic kidney diseases.

Among the newest promising kidney therapies, bardoxolone-methyl, an anti-oxidant agent, was first reported as a successful treatment in improving kidney function of chronic kidney disease patients (phases IIa clinical Bardoxolone Methyl Treatment: Renal Function in CKD/Type 2 Diabetes - BEAM trial) (Pergola *et al.* 2011). Although these encouraging effects have been shown, a more recent study pointed out that risks of heart failure were increased by bardoxolone as well as non-fatal myocardial infarction, non-fatal stroke, hospitalization for heart failure, or death from cardiovascular causes, which led to termination of the trial (de Zeeuw *et al.* 2013). We chose bardoxolone for characterizing further the ZSF1 rat model of chronic kidney disease and diabetic nephropathy and compared its effects head to head with standard of care lisinopril (ACEi).

Mild diabetic and renal deficient male ZSF1 rats (12w old) were unilaterally nephrectomized for further deteriorating kidney function. Bardoxolone or lisinopril treatment was administered as food admix for 8 weeks from 22 weeks of age (*i.e.* 10 weeks after UNx was performed). Kidney function of

placebo- or drug-treated UNx-ZSF1 rats was evaluated by quantifying selected panel of biomarkers in plasma and urine. Kidney Injury Molecule (KIM-1) has been added to the biomarkers list previously mentioned. KIM-1 is a transmembrane protein expressed by tubule epithelial cells in response to injury, and has been described as a biomarker of proximal tubular injury in both Human and rodents (Han *et al.* 2002; Lock 2010; Fuchs and Hewitt 2011; Kirk 2013; Luk *et al.* 2013). At the end of the treatment, kidney function was further evaluated by glomerular filtration rate determination and kidney structure was examined by immunohistological methodology.

Experimental study 3 (*Pflügers archiv*, to be submitted in March)

Bardoxolone methyl decreased body weight, improved kidney function in male unilateral nephrectomized ZSF1 rats associated with specific gene changes and signs of toxicity

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Key words: Chronic kidney disease, ZSF1 rats, Bardoxolone, Mechanism of action, kidney function.

Running title: Bardoxolone methyl improved kidney function in ZSF1 rats.

Abstract

Current treatments are ineffective in reversing the progression of chronic kidney disease (CKD) [1] in patients. In clinical studies in CKD patients, Bardoxolone methyl (BARD), an anti-oxidant agent, improved kidney function but with increased cardiac and hepatic side-effects. In rodent models of diabetes and renal disease, BARD or its analogues were reported to improve kidney function, except in ZDF rats wherein BARD analogues worsened diabetic nephropathy and induced liver side-effects. We employed male ZSF1 rats as a model of metabolic syndrome and CKD to investigate the effects of chronic treatment with BARD in direct comparison to Lisinopril (Angiotensin Converting Enzyme inhibitor, ACEi). BARD reduced body weight and food intake, improved kidney function, kidney biomarkers and normalized metabolic parameters without effects on blood pressure. Lisinopril improved kidney function to an extent similar to BARD, via reduced blood pressure. Both treatments similarly decreased glomerular and tubular damage as assessed by histological score. To test whether or not the food intake reduction induced by BARD could explain its effects, a second experiment was performed in untreated, food restricted animals. Food restriction improved kidney function similarly to BARD, however, gene expression analysis in kidney revealed that BARD differentially regulated genes compared to lisinopril or food restriction. Nrf2-regulated pathways were significantly up-regulated by BARD, while neither lisinopril nor food restriction showed specific pathway-level regulation. Our data suggest that the effects of BARD in ZSF1 are driven partially by body weight loss and partially by induction of Nrf2-responsive genes, and are independent of blood pressure reduction.

Introduction

Type 2 Diabetes (T2D) has been recognized as a growing worldwide epidemic. The World Health Organization predicts that the number of people with T2D will increase from 170 million today to between 284 and 347 million in 2030 [2], [3]. A major comorbidity of T2D is Diabetic Nephropathy (DN) which is the leading cause of Chronic Kidney Disease (CKD) [1] and End-Stage-Renal Disease

(ESRD) [4]. The etiology of CKD is multifactorial with underlying mechanisms that are not fully understood [5]. CKD patients are at elevated risk for cardiovascular events [6], [7].

Hyperglycemia, oxidative stress, inflammation and hypertension [8], [9] are factors that accelerate development of CKD. Impairment of endothelial and vascular function triggers development of DN and contributes to loss of kidney function at all stages of CKD [10]. The underlying pro-inflammatory state attributed to obesity often found in these patients, has also been implicated in CKD development both in animal models [11] and in patients [12], [13], [14].

CKD patients demonstrate disturbances in blood pressure regulation due to alterations in the Renin Angiotensin Aldosterone System (RAAS) [15], [5]. Reducing hypertension with angiotensin converting enzyme inhibitors (ACEi) improves CKD [16] by reducing both systemic and glomerular pressure, improving glomerular filtration. ACEi may also have anti-inflammatory effects [14], [17].

In contrast, Angiotensin Receptor Blockers (ARBs: Losartan, Valsartan, Telmisartan, etc.), improve kidney function mainly by reducing albuminuria [18], [19], as well as reducing blood pressure and improving metabolic profile [20]. Combinations of ARBs and ACEi have shown additive protective effects in remnant kidney models in rat [21], suggesting that targeting several points in the RAAS simultaneously may be more beneficial, although the benefits of combination therapy in CKD patients remains controversial [22], [23].

Other drugs with potential for the treatment of CKD include the direct renin inhibitor aliskiren, which improved DN in mice when combined with losartan [24]. In addition, mineralocorticoid receptor antagonists including spironolactone and eplerenone have been shown to be renoprotective in patients [25] and in animal models [26], [27] via modest effects on blood pressure, as well as reduction of oxidative stress, fibrosis and inflammation [28].

It is clear that tackling DN will require drugs targeting pathways beyond the RAAS [29]. The anti-inflammatory drug bardoxolone methyl (BARD) is an activator of Nrf2 and Keap-1 [30] and has been evaluated in animal models of CKD and clinical studies in patients. Nrf2-knockout female mice developed severe glomerulonephritis associated with severe oxidative stress [31]. In preclinical

studies, BARD or its analogues improved kidney function in cisplatin-induced-nephrotoxicity in mice [32] and in an acute kidney injury mouse model [33]. In both chronic rat models of kidney failure induced by 5/6 nephrectomy [8], and long-term treatment in ZDF and STZ rats, DIO mice [34], [35] or cynomolgus [36], BARD improved kidney structure and function. In one study performed in ZDF rats, treatment with high doses of BARD analogues worsened diabetic nephropathy, increased albuminuria and induced other side-effects [37].

The clinical experience with BARD has been mixed. BARD improved kidney function during short term studies in moderate to severe CKD patients with T2D [38], [39], [40], [41] despite increasing albuminuria [38]. More recently, in a Phase 3 trial, BARD did not improve outcomes in ESRD stage 4 CKD patients with T2D but was instead associated with increased incidence of cardiovascular death [42]. These results have stimulated a reassessment of the therapeutic potential of BARD in CKD as well as other indications [30], [42], [43].

In this report, we investigated the effects of BARD during chronic treatment in the uninephrectomized male ZSF1 rat, a model of T2D and CKD. The effects of BARD were evaluated vs. lisinopril (ACEi) or food intake restriction, by measuring kidney function (glomerular filtration rate, GFR), selected biomarkers, histological examination, and kidney gene expression.

Materials and Methods

Animals

Experimental procedures were performed under an animal research protocol (ARP) authorization provided to F. Hoffmann-La Roche by the Swiss Cantonal Veterinary Office Basel-Stadt. Studies were performed in accordance with guidelines of the Association for the Assessment and Accreditation of Laboratory and Animal Care (AAALAC # Unit Number: 001057) and appropriate federal, state and local guidelines. Male ZSF1 rats [(OB) Gmi Crl-fa/fa] were ordered at the age of

10W from Charles River U.S.A. and fed a special diet equivalent to PURINA PMI 5008 (Ssniff R/M Purina 5008 mod. 10mm pellet (proteins 38kJ%, carbohydrate 45kJ%, fat 17kJ%) as recommended by the animal provider to accelerate development of metabolic disease. Rats were group-housed (4 per cage) at approximately 21°C and 55–65% relative humidity and had ad libitum access to food and water. Light cycle was from 6 a.m. to 6 p.m.

Surgery

At the age of 12W, rats underwent unilateral nephrectomy [44]. Animals were treated with Buprenorphine (0.05mg/kg, subcutaneous s.c.) one hour prior to surgery to minimize pain. Rats were anesthetized with isoflurane/O2 and the left flank of the animal was shaved and the skin was washed with Betadine solution. The rat was placed upon a heated operating surface to maintain rectal temperature at 37°C. The left kidney was accessed via a small lateral flank incision and exposed allowing the access to the renal pedicle. The vascular pedicle was dissected free from adipose and occluded with a titanium clip (Braun Aesculap) followed by removal by excision. The flank incision was sutured (Ethicon perma-hand 4-0, 19mm 3/8 circle) and the skin was closed with atraumatic clips (3M- DS 25 Precise disposable skin stapler). Each animal was placed back in its cage and single housed for at least one week to allow full recovery. Each animal received Buprenorphine (0.05mg/kg/day, subcutaneous, s.c.) for the two days following surgery in order to minimize pain. After the wound had completely healed, the skin staples were removed and the animals returned to group-housing.

Drug treatments

At 22 weeks of age, 10 weeks following nephrectomy, animals were randomized to treatment groups (N = 9-10 animals per group) according to body weight and a selection of urine biomarkers of kidney function (Albuminuria (Alb), proteinuria (Prot) and Cystatin-C (Cyst-C)). Compounds were administered as food admix in PURINA PMI 5008 modified food for eight weeks.

The food admixes were prepared by Ssniff Spezialdiäten GmbH (Soest, Germany) assuming daily food consumption of 33-35g/animal. Bardoxolone methyl (Atlanchim Pharma, France) was administered at a dose of 10mg/kg/d and Lisinopril (Small Molecules Inc., Hoboken, NJ, USA) was administered at a dose of 30mg/kg/d. In a separate cohort of rats (study 2), a food restricted group was fed with 10g of PMI 5008 daily which corresponded to the average food intake per day under BARD treatment in Study 1. The impact of food restriction on metabolic and renal parameters in Study 2 was compared to its own vehicle group which has ad libitum food access.

Blood pressure monitoring

Systolic and diastolic arterial blood pressure (SAP, DAP) measurements were performed toward the end of the treatment period i.e. after completion of 7 weeks of dosing. Blood pressures (BP) were measured in conscious animals with a computerized tail-cuff system, based on the Volume Pressure Recording technology (Model CODA; Kent Scientific Corporation, USA). SAP and DAP were measured 2 hours after the animals had been transferred a dedicated room heated to 28°C. For each rat, a cycle of 10 mock BP readings was applied, before 10 BP measurements were performed. At least 8-10 sequential BP measurements were obtained to derive valid SAP and DAP values.

Blood and urine sampling and biochemical assays

Rats were placed into metabolic cages with ad libitum access to water and food and urine was collected over 24h, once at baseline, prior to treatment start, then every 4 weeks thereafter. The urine samples were maintained at 4°C during collection with a chiller. Samples were centrifuged at 800 RPM for 10 minutes and supernatants were used for analysis.

Blood was collected in the morning from 4h-fasted animals in EDTA-coated tubes at necropsy following decapitation under isoflurane anesthesia. Plasma was recovered following centrifugation at 4°C (10,000 RPM) for 15 minutes, and stored frozen (-20°C) for bio-analytical analysis. Metabolic and enzymatic profiles were measured on a COBAS 6000 system using the following kits: Triglycerides (Roche Diag. 20767107 322), Cholesterol (Roche Diag. 03039773 190), non-esterified fatty acids (NEFA, Roche Diag. 11383175 001), plasma glucose (Roche Diag. 04404493 190), Creatinine (Roche Dig. 03263991 190), Fructosamine (Roche Diag. 04537939 190); alanine aminotransferase (ALT, Roche Diag.20764957 322) and aspartate aminotransferase (AST; Roche Diag. 20764949 322)].

Commercially available rat enzyme-linked immunosorbant assay (ELISA) kits were used for measurement of plasma Cystatin-C (R&D Systems, MSCTC0), plasma Insulin (rat/mouse insulin FIA, Mercodia®, 10-1248-10), urinary Albumin (Alpco, 41-ALBRT-E01), urinary monocyte chemo attractant protein-1 (MCP-1, R&D Systems, SMJE00) and urinary transforming growth factor beta-1 (TGF-β1, BioLegend, 436706). Determination of plasma Osteopontin (OPN), kidney injury molecule-1 in urine and plasma (KIM-1), urinary β2-microglobulin (β2M), urinary Cystatin-C (Cyst-C), and urinary Neutrophil gelatinase-associated lipocalin (NGAL) was done using multiplex assays (Millipore) performed according to the manufacturer's instructions. The concentrations of urinary biomarkers were normalized to urine volume.

Body fat content determination by Magnetic Resonance Relaxometry (MRR)

Magnetic resonance relaxometry (MRR) [45] was performed in study 2, to determine body composition (fat mass, lean mass) at 2 time points (+3 week, +7 weeks after food restriction start). In brief, shortly before the MR investigations, individual awake animals were transferred into 1200mL polyethylene containers with holes for air circulation. The containers were closed with screw-top lids and then placed in the MR scanner (Biospec 47/40, Bruker, Ettlingen, Germany). Hydrogen T2-MR relaxometry was accomplished at 4.7 Tesla using a multiple spin-echo sequence with 90° excitation and 256 refocusing pulses, 2.5ms echo time, 256 sample points, 2 averages and 10s repetition time. After the examination, lasting for less than 2 minutes in total, each animal was weighed and returned to its cage.

The raw data was subjected to an inverse Laplace transformation. The resulting relaxograms revealed distinct and well-separated peaks for tissue water (T2: 10-99ms), fat (T2: 100-400ms), and free water (T2: >400ms). On the basis of well-established calibration factors (water proton density: 9.01g/mol protons, average proton density of triglycerides: 8.59g/mol protons), areas under the curves were determined and converted to total body fat and body lean mass in grams.

Measurement of Glomerular Filtration Rate

Measurement of glomerular filtration rate (GFR) was performed 1-week before the end of the treatment period using the Fluorescein isothiocyanate (FITC)-Inulin clearance method [46]. FITC-inulin (Sigma–Aldrich, St. Louis, MO, USA; cat no F3272, 40mg/mL in 0.9% NaCl) was injected (1mL/kg, i.v.) and blood samples (via tail nick, volume ~100µL each) were collected in EDTA-coated tubes (Microvette 300µL, SARSTEDT, D-51588 Nümbrecht, Germany) at +0.5, 3, 7, 10, 15, 20, 40 and 60 min after inulin injection. The samples were kept on ice. Plasma, generated as described above, was stored in 96 well plates at -20°C before measurement of inulin. GFR was calculated for each individual rat then averaged for each treatment group.

Measurement of FITC Inulin in plasma: Because pH significantly affects FITC florescent values, plasma samples were buffered to pH 7.4 with 500mM HEPES. To 40µL of HEPES buffer in each well of a 96- well plate (Costar, #3694), 10µL plasma were added and mixed. Fluorescence was determined using a microplate reader (Infinite M200 Pro, Tecan) with 485nm excitation and read at 538nm emission [47].

Organ preparation and histology

Animals were sacrificed and kidney, heart and liver were harvested and weighed. Hearts and livers were then immediately immersed in 10% neutral buffered formalin for 24h. Eyes were removed and fixed in Davidson's fluid for 24h. Each kidney was divided in two pieces. The first kidney half was placed in 10% neutral buffered formalin for 24h); the second half was dissected from its medulla and cortex and stored in RNAlater® solution (Ambion, CA, USA) for later RNA isolation and gene expression analysis.

Tissues were removed from formalin or Davidson's fluid, rinsed in PBS, embedded in paraffin blocks, and sections of 4µm were prepared. Sections were stained with hematoxylin eosin (HE; all tissues) or Periodic acid-Schiff (PAS; kidney and eyes). For the detection of macrophages in the kidney, de-paraffinized sections were stained with the Ventana DAB Map TM kit using a mouse monoclonal anti-CD68 antibody (1:100; Abcam, ab31630) and detected with a biotinylated donkey-anti-mouse secondary antibody on a Ventana Discovery XT immunostainer (Ventana medical System, Tucson, Arizona). Similarly, kidneys were stained for the detection of lymphocytes with a polyclonal rabbit-anti-CD3 antibody (1:100; DAKO, Ao452) detected with a biotinylated donkey-anti-rabbit antibody. Immunohistochemically stained slides were scanned and the stained marker area was quantified using the Definiens Tissues Studio 3.6 software.

Histological scoring was assigned for kidney structure changes (glomerular vacuolation, thickening of basement membranes, interstitial inflammation, interstitial fibrosis, tubular dilation, proteinaceous casts) as follows: grade 0 (no change), grade 1 (minimal; ≤ 10% of a structure affected), grade 2

(slight; between 10 and 20% of a structure affected), grade 3 (moderate; between 20 and 50% of a structure affected), grade 4 (marked; between 50 and 75% of a structure affected), and grade 5 (severe; up to 100% of a structure affected).

For glomerulosclerosis (GS), 50 glomeruli per kidney were examined and each scored into grade 0 (no GS), grade 1 (≤ 25% of glomerulus affected), grade 2 (≤ 50% of glomerulus affected), grade 3 (≤ 75% of glomerulus affected), and grade 4 (up to 100% of glomerulus affected). To calculate a GS score for each kidney, the sum of all grades from the 50 glomeruli was calculated. The obtained sum score was graded as follows: grade 1 (sum ≤ 25), grade 2 (sum between 26 and 50), grade 3 (sum between 51 and 75) grade 4 (sum between 76 and 100) grade 5 (sum >100).

Determination of plasma drug levels

Plasma exposure for BARD was determined from samples collected at the end of the study using a quantitative LC/MS/MS method.

Statistics

Each study (study 1: Vehicle, BARD, Lisinopril; study 2: Vehicle, food restriction, Lisinopril) was analyzed separately. General parameters (body weight BW, food intake FI, organ weight), plasma and urine parameters and GFR of treated-UNx-ZSF1 rats were compared to respective vehicle group. Values were expressed as mean ± SEM. ANOVA was used followed by post-hoc Dunnett's test with significance *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to vehicle.

Kidney RNA extraction and gene expression analysis

Frozen kidney tissue in RNA later was homogenized using a Tissue Lyser (Qiagen, Germany). A single steel bead and 20mg of cortex stored in RNA later® solution (Ambion, CA, USA) were added

to 1ml of RLT-plus Lysis buffer, put on ice and homogenized for 2 x 2 Min at 20Hz, followed by 2 Min centrifugation. RNA was extracted from 400µl supernatant using the QIAsymphony kit (19585, S-Blocks 96) with final elution volume of 100µl.

To prepare fragmented, labeled cDNA for microarray hybridization, 50ng of total RNA were subjected to the NuGen Ribo-SPIA TM amp (NuGen, CA, USA) protocol to synthesize first and second strand cDNA followed by Amplification with the SPIA protocol according to manufacturer's instructions. Then, 3µl of Ovation TM WB reagent were added to 79µl of SPIA reaction and incubated at 48°C for 30 Min. Amplified cDNAs were then purified using the QiaQuick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's instructions and eluted in a volume of 30µl. Fragmentation and biotin labelling were then performed using the Encore Biotin kit (cat. 4200; NuGen, CA, USA) with 4.4µg cDNA in 25µl buffer, mixed with 7µl of the Fragmentation Master Mix and 18µl of the Labelling Mix according to the manufacturer's instructions. Selected samples were quality checked for extent of cDNA fragmentation on an Agilent Bioanalyzer.

For Microarray hybridization, the HWS kit (Affymetrix, UK) was used with 50µl of fragmented, biotinylated cDNA according to the manufacturer's instructions. Hybridization, Washing and Staining were performed using the respective Affymetrix kit (cat. 900720), Rat Genome 230 2.0 arrays and a Fluidics-450 station.

Statistical and bioinformatics analyses of GEP

Kidney samples (N=57) were profiled with Affymetrix RAT 230 2 chip (Santa Clara, CA). Quality control (QC) was performed with an internal software pipeline. Samples with quality issues (N=7) were removed from subsequent analysis. Signal intensities of the remaining 50 chips (Vehicle control: N=17; Lisinopril: N=15; Food restriction: N=9; BARD: N=9) were normalized by the RMA method as implemented in the affy package in R/Bioconductor [48].

Differentially expressed genes were identified by linear models and the empirical Bayes method implemented in the limma package [49]. Both the treatments, as well as RNA degradation slope were

used as covariates since considerable variability was observed. Post hoc analysis revealed that applying a correction for RNA degradation substantially improved the model (tested by ANOVA and visual inspection of p-value distributions, data not shown). Contrasts of the linear model [50] included: (1) Lisinopril, food restriction, and BARD vs. vehicle respectively, [50] BARD vs. food restriction ('BARD-net'), and (3) BARD-net vs. Lisinopril.

Gene expression pathway analysis was performed with both the Gene Set Enrichment Analysis (GSEA) algorithm [51] and the camera method [52], which gave concordant results. Gene sets were derived from an internal database that integrates public data including the Reactome database [53] and the NCI pathway interaction database [54]. For both gene-level and pathway-level analysis, multiple-testing adjustment was performed with the Benjamini-Hochberg method to control for false-discovery rates [55].

Results

Survival, body weight, food intake and diuresis

At the age of 24 weeks, rats weighed approximately 620g. Group of rats were randomized into treatment groups based on BW and selected kidney parameters (Alb, Prot and Cyst-C). The BWs in the vehicle-treated group increased continually reaching about ≈720g after 11 weeks of treatment (i.e. +15% BW gain). No mortalities occurred in either study. In both studies, food intake (FI) averaged 33g/ day (as measured weekly) in vehicle groups and remained constant during the treatment period. Lisinopril showed no effect on FI with a trend toward reduced rate of BW gain (-12% (NS) vs. vehicle). In contrast, BARD strongly reduced food intake by 46% after the first week of treatment (38.6g ±1.3 (at baseline) to 21.0g±0.7 week 1) (Figure 1). The FI reduction in the BARD-treated group continued to decrease, plateauing at the second week of treatment. At the end of the treatment period, animals in the BARD group were consuming about 10.0g±0.0 food per day, corresponding to a 75% reduction

in FI vs. vehicle. The dramatic FI reduction induced by BARD resulted in decreased BW gain of 32% compared to Vehicle. Diuresis was increased by 45% by lisinopril while BARD significantly reduced diuresis by 68% vs. control animals.

Effects on blood pressure

Lisinopril significantly reduced mean arterial pressure (MAP) after 7 weeks of treatment by 28% compared to vehicle (Figure 2). The hypotensive effect of Lisinopril in ZSF1 rats resulted from similar reductions in both DAP and SAP (data not shown). Treatment with BARD had no effect on MAP.

Plasma parameters and kidney biomarkers

After 8 weeks of treatment, lisinopril showed a tendency to reduce plasma Creatinine (pCre reduction by 13% vs. vehicle) and significantly improved plasma KIM-1 (pKIM1 reduction by 66% vs. vehicle) (Figure 3). BARD significantly reduced pCre (by 56%, $p<0.001$) and pKIM1 concentrations (-35%, $p<0.01$) compared to vehicle. The effect of BARD to reduce pCre was 3-fold greater compared to the effect of lisinopril (-56% vs. -13%).

At the end of the study (i.e. at 32w of age), control ZSF1 rats were hyperglycemic, dyslipidemic and hyperinsulinemic (Table 1). Lisinopril tended to exacerbate hyperglycemia but had no effect on insulin and strongly decreased lipid levels. The increase of plasma glucose induced by lisinopril has been reported mainly in patient care and health information (See Mayo Clinic and in other health care recommendations in supplemental web links available at the end of the references). BARD significantly reduced and normalized all metabolic parameters except total cholesterol (TC) which remained unchanged (Table 1).

Effects on organ weight and liver histology

Organs weights were expressed both as a percentage of body weight (% BW) (Table 2), and as absolute values (Table 3). Lisinopril tended to reduce liver weight and fat mass (NS). Consistent with the significant body weight loss in the BARD group, the weights of all organs, except for liver, were also reduced. BARD treatment resulted in substantial increases in liver weight (from 3.67g±0.14 in vehicle to 16.48g±0.76 in BARD-treated rats). Reduction of organ weight induced by BARD (Table 3) was not fully driven by BW loss since the extent of these effects persisted when organ weight changes were expressed relative to body weight (Table 2).

Changes in liver induced by BARD were associated with modestly increased alanine transaminase and aspartate transaminase (ALT and AST) levels (ALT: +55%, AST: +63%; data not shown). BARD did not change the gross appearance of other organs (kidney, heart). After 8 weeks of treatment with BARD, however, the livers had a milky appearance, were grossly larger and weighed more than in vehicle-treated groups (Table 3). Livers from control, untreated ZSF1 rats were characterized by hepatocellular vacuolation and rarefaction (interpreted as increased glycogen storage) (Figure 4A-B). Livers from BARD-treated animals showed reduced hepatocellular vacuolation compared to vehicle animals and animals treated with lisinopril. Furthermore, BARD tended to reduce hepatocellular glycogen storage (Figure 4). However, BARD tended to increase bile duct hyperplasia.

Effects on kidney function

Kidney function was determined at the end of treatment using the inulin clearance method. The GFR in control animals was extremely low compared to healthy rats (GFR=0.55±0.10 ml/min/g kidney) (Figure 5). In lean, aged-matched ZSF1 rats, GFR is approximately 4-to-5 ml/min/kg (in-house data). Lisinopril treatment increased GFR by 78% (Figure 5A) while BARD significantly increased GFR vs. vehicle by 237% (Figure 5).

The urine albumin / creatinine ratio (uACR) was elevated in control ZSF1 rats at the end of treatment. This elevated ratio was mainly driven by increased Alb as the changes in urine creatinine were minimal (in-house data). uACR was significantly reduced by Lisinopril treatment (Figure 5B). BARD significantly reduced uCre (-77%) and Alb (-61%) (Figure 6A) compared to vehicle, leading to an unchanged uACR (Figure 5B).

From 4w of treatment, lisinopril significantly decreased urine albumin and KIM-1 levels (Figure 3). The effects were greater after 8w of treatment. Lisinopril also modestly reduced Cyst-C and β2M after 4W, effects that were more pronounced after 8 weeks of treatment, reaching significance vs. untreated animals. In contrast, BARD had no effect on these 4 selected biomarkers after 4 weeks, but significantly decreased urine Alb, KIM-1 and Cyst-C excretion (Figure 6B-C) after 8 weeks to a comparable extent as lisinopril. In contrast to the effect of lisinopril, BARD strongly increased β2M excretion levels after 8 weeks of treatment (Figure 6D).

Effects on inflammation markers

Inflammatory markers were low at baseline (time 0, *i.e.* when rats were 24W-old). Plasma TGF-β1and MCP-1 progressively increased during disease development. Lisinopril tended to decrease TGF-β1 after 4 weeks of treatment but had no effect after 8 weeks (Figure 7A). Lisinopril reduced MCP-1 levels after both 4- and 8-weeks of treatment (Figure 7B). BARD reduced TGF-β1 after 8 weeks of treatment but significantly increased MCP-1 by more than 2-fold after 4 weeks of treatment.

Effects of bardoxolone on food consumption behavior

BARD was administered orally as food-admix at an intended dose of 10mg/kg/day. Since food intake in BARD-treated rats was reduced from 33g to 10g/day, we should assume that rats were treated with BARD@3mg/kg/d. Plasma exposure of BARD was 2.3±0.2ng/mL as determined at the end of the treatment period after a 4-5 hour fast. The measured exposure of BARD in this study corresponds to plasma exposure in patients 48h after last treatment at the 900mg/day dose, based on the pharmacokinetic profile in human [56] and rodent (in-house data).

Due to the significant impact of BARD on food consumption, we evaluated its impact on feeding behavior. A chain pull assay was performed in a separate cohort of trained Lister Hooded rats. Acute treatment with BARD did not change their eating motivation (not shown) suggesting that BARD did not confer taste aversion to the diet.

Effects on organ histology

At the end of the treatment period, vehicle animals were characterized by presence of glomerulosclerosis (GS) (Figure 8) and other histopathological alterations in the kidney (Figure 9). Lisinopril improved GS, mainly by reducing the number of glomeruli with grade 1 and grade 2 and increasing the number of healthy glomeruli (grade 0). BARD primarily reduced the number of glomeruli with grades 1 and 4 but had no effect on other grades of GS.

Lisinopril also reduced glomerular basement membrane thickening, and reduced interstitial fibrosis, tubular dilation and proteinaceous casts. Treatment with BARD also reduced basement membrane thickening, interstitial inflammation, interstitial fibrosis, tubular dilation and proteinaceous casts. Overall, BARD and lisinopril showed comparable improvements in kidney histological scores (Figure 9 and Figure 10).

Effects on inflammatory infiltrates in kidney (macrophages and lymphocytes)

Inflammatory cells in the kidney were identified by immunohistochemistry with antibodies directed against CD68 (macrophages) and CD3 (lymphocytes). BARD reduced inflammatory infiltrates (CD68 (Figure 11B) and CD3 (Figure 11E) compared to vehicle. The decrease in macrophages in BARD-treated kidneys was greater than in lisinopril-treated kidneys and (Figure 11C) comparable to the effects of food restriction (Figure 11D).

Impact of bardoxolone on heart and eye structure

In ZSF1 rats at the end of the treatment (i.e. at the age of 32 weeks), only minimal histopathological findings were present in the heart. These included arterial thickening, interstitial fibrosis and inflammatory infiltrates as well as macroscopic cardiac hypertrophy. There were no differences in incidence and severity of findings in vehicle animals and animals treated with lisinopril and BARD (data not shown).

Similarly, only minimal histopathological findings were present in the eye in individual animals. These included hydropic swelling of lens cells, as well as hyperplasia of the lens epithelium as sign of a cataract, most likely secondary to the diabetic condition of the animals. Other parts of the eyes, including the retina, showed no histopathological findings. There were no clear differences in incidence and severity of the cataract in vehicle animals and animals treated with Lisinopril or BARD (data not shown).

Impact of food restriction on body composition (MRS), blood pressure, kidney function and kidney biomarkers

The substantial effect of BARD on food intake prompted us to independently assess the effects of food restriction to a similar degree in a second cohort of UNx_ZSF1 rats. Food was restricted to 10.0g/d to match the food intake under BARD treatment (9.4g±0.4). BW gain reduction under food restriction was 25% (Figure 16A) which was comparable to the extent of BW loss induced by BARD (Figure 1). Food restriction strongly improved multiple metabolic parameters (Table 4). Reduction of glucose, insulin and triglycerides induced by food restriction (Table 4), was superior to the effects of lisinopril treatment. Reductions in NEFA and total cholesterol in food restricted rats was similar to the effects of Lisinopril. MRS analysis of body composition showed that food restriction reduced both lean mass and fat mass to similar extents (Figure 12A).

Food restriction improved kidney histology (Figure 10D) and decreased macrophage infiltration (Figure 11D). Food restriction similarly improved GFR by +319% vs. vehicle as observed for BARD (+237% vs. its own vehicle). This improvement was achieved without change in MAP (Figure 16C). When comparing the two cohorts of rats treated either with BARD or under food restriction, we observed comparable improvement in urine biomarkers (BMs) of kidney function; for food restriction: Alb (-63%) and KIM-1 (-87%) vs. its vehicle and for BARD: Alb (-61%) and KIM-1 (-69%) vs. its vehicle (Figure 17A&B). Of note, improvement in Cyst-C was superior under food restriction (-82%) compared to BARD treatment (-31%). Food restriction had no effect on β2M or MCP-1 (Figure 17D&F), in contrast to the effects of BARD (Figure 7 and Figure 6), suggesting some specific effects of BARD on top of FI reduction. In addition to improvement in kidney markers, food restriction improved kidney structure similarly to the effects of BARD, i.e. reduced basement membrane thickening, interstitial inflammation, tubular dilation, interstitial fibrosis, proteinaceous casts (not shown) and GS (Figure 18).

Effects of treatments on gene expression

To further explore the contribution of BW reduction to the improvement of kidney function under BARD treatment, we examined the gene expression signatures in kidney in each treatment condition. The schematic in Figure 13 represents global mRNA expression profiles. The results demonstrate that the gene expression changes in response to food restriction only partially overlap the response to BARD. The effects of lisinopril appear intermediate between the profiles of vehicle controls and food restricted animals. As expected, BARD significantly up-regulated several established targets of Nrf2 including Nqo1, Gsta2/3, and Keap-1, when compared with vehicle control or food restricted animals. On the pathway level, BARD specifically modulated gene sets in established biological networks downstream of the Keap1-Nrf2 axis, including pentose phosphorylation (Figure 14A), glucuronidation (Figure 14B), heme degradation (Figure 14C) and glutathione conjugation (Figure 14D) pathways (FDR<1E-4 compared with Lisinopril or food restriction).

Discussion

The currently available marketed drugs targeting the renal and systemic microvasculature, inflammation and insulin resistance are insufficient in treatment of chronic kidney disease [57]. A major limitation in identification and development of new therapies may be the complex etiology of the disease and our incomplete understanding of the pathways and targets involved in the loss of kidney function and the development of CKD [58]. It has become apparent that tackling CKD will require a multi-factorial approach that treats the disease, not as an isolated pathology, but within the broader context of vascular dysfunction, inflammation and metabolic disease. Progress on the research front is oven hampered by the unavailability of rodent models of metabolic syndrome and CKD closely resembling human CKD. Recently, several new mouse models of CKD have been developed and characterized [59], while no completely suitable rat models have yet been developed.

We explored the male ZSF1 rat as a model to investigate the effects of chronic treatment with Bardoxolone methyl on renal and metabolic function. The male ZSF1 rat reflects many aspects of metabolic syndrome in human including obesity, glucose intolerance, dyslipidemia, insulin resistance [60], [61], hypertension [62] and features of kidney dysfunction [60]. We performed renal mass reduction by UNx [44] to accelerate the development and severity of renal histological and functional defects. Indeed, it has been demonstrated previously, that renal ablation or induced renal dysfunction on a diabetic background increases the rate of renal dysfunction [63], in part by accelerating hyperfiltration in the remnant kidney [17].

In this study, adult ZSF1 rats were treated starting at 22 weeks of age 10 weeks after UNx was performed. The treatments were given as a foodamix in order to prevent stress from repetitive animal handling. Lisinopril and BARD were administered at previously evaluated pharmacological doses in rodents. For BARD, the dose chosen (10mg/kg/d) was comparable to studies performed in rodents [33] or monkeys [36]. Both treatments were well tolerated except induction of liver findings (Figure 4). In control, untreated animals, livers were characterized by hepatocellular vacuolation and rarefaction. Under BARD treatment, both the absolute liver weight and liver weight/BW ratios increased compared to vehicle (Table 2) with evidence for bile duct hyperplasia (Figure 4). The liver histological findings under BARD treatment correlated with a modest increase in ALT and AST enzyme levels (+55% and +63% versus vehicle, respectively). Liver findings in BARD-treated UNx-ZSF1 although not similar to that observed in ZDF rats [37] or in patients [64], [30], [43], would confirm a potential risk for liver toxicity. For BARD, no mortalities occurred (as opposed to those observed in ZDF rats treated with BARD analogues [37] and no adverse behavioral effects were noted during treatment. Histological examination post-mortem, revealed no changes in heart, kidney or eyes.

BARD treatment was associated with a rapid and sustained reduction in food intake that led to reduced rate of body weight gain and increased diuresis (Figure 1). The improvement of metabolic values (Table 1) is likely due to the effects on body weight, since a food restricted group showed similar improvements in metabolic parameters. The improved renal function (GFR increase) in the

BARD group may also be partially due to the effects on body weight. Indeed, studies have shown improved eGFR, renal outcomes and reduced cardiovascular events [65] in obese patients that have experienced dramatic weight loss following bariatric surgery (see review [66]). Various dietary regimens that reduce body weight, have also been associated with reduced blood pressure and improved insulin sensitivity, which may positively affect renal function [67]. The improvement of kidney function in ZSF1 rats treated with BARD in our study was comparable to the improvement induced by food restriction or by lisinopril treatment (which did not affect BW). These factors suggest that part of the improvement in kidney function by BARD was driven by reduction of body weight, although we could clearly identify gene signatures suggesting that Nrf2 pathway was altered in BARD-treated kidney vs. kidneys from the food restricted animals. Although, the impact of BARD on BW cannot be prevented and discriminated from the activation on Nrf2 pathway either in animal model [37] or in patients [42], our data suggest that BARD improvement of kidney function result from shared mechanisms on BW and Nrf2 pathway activation. Only a forced feeding (by gavage) of UNx-ZSF1 rats treated with BARD would allow abolishing impact of BARD on BW loss. Even under this hypothesis, the stress induced by chronic and forced gavage could generate an additional biased factor.

Effects of BARD on food intake have been previously noted in both patients enrolled in the BEACON trial [42] and in rats [37]. Studies of the pharmacokinetic behavior of BARD in patients [56] suggest that the observed hypophagia is unlikely through satiety signals since BARD is not found at appreciable levels in the brain. The reduction in food intake in our study appears not to be explainable by altered palatability, since rats consumed the diet immediately upon presentation, without signs of aversion. Altered energy balance or increased energy expenditure could be an explanation to the food intake reduction induced by BARD, although this was not investigated.

The effects of BARD to improve renal function were independent of blood pressure reduction since no change in systemic blood pressure was observed during treatment. In contrast, improved kidney function under lisinopril treatment can be attributed, in part, to its blood pressure-lowering effects.

Plasma KIM-1 levels were significantly reduced by all treatments, suggesting an improvement of proximal tubular function [68], consistent with previous observations in patients [69]. BARD, food restriction or lisinopril reduced urinary albumin excretion to similar extents. Daily urinary excretion of other kidney BMs (Cyst-C and β2M, markers of tubular function and glomerular filtration, respectively) were also evaluated (Figure 6). Similar to the effects observed in food restricted or lisinopril-treated rats, BARD significantly reduced urine Cyst-C, suggesting improvement of tubular function [70] and reduction of CKD progression [71]. BARD reduced TGF-β1 to a greater extent than did lisinopril (Figure 7B). While lisinopril reduced urine β2M and MCP-1 levels (Figure 6 and Figure 7), BARD increased them modestly (β2M) (Figure 6D) or significantly (MCP-1) (Figure 7B). Despite the absence of improvement in these particular parameters by BARD, in contrast to the positive effects of lisinopril and food restriction, histological evaluation showed improved renal morphology (Figure 9 and Figure 10) confirming its renal protective effect. Despite an increase in urinary MCP-1 excretion, BARD tended to reduce the number of kidney macrophages (CD68) and lymphocytes (CD3), similar to previous data (Figure 11B&F) in CKD patients [14]. It is worth noting that BARD treatment or food restriction similarly reduced kidney macrophages and lymphocytes and to a greater extent than did lisinopril. In summary, our data demonstrate that BARD protects kidney structure and function and delays CKD progression in the ZSF-1 rat model. The increase in MCP-1 and TGF-β1 induced by BARD are inconsistent with the improvement in kidney function (GFR, uACR) and morphology (histology, staining for CD68). Despite improvement of kidney function and structure by BARD, increased urinary MCP-1 and TGF-β1 concentrations and change in liver structures could indicate raising toxicity. Further investigation would be needed to understand the significance of the increase in MCP-1 and TGF-β1 induced by BARD that could provide some explanation regarding the failure of BEACON [72]. In this regard, data collected in UNx-ZSF1 rats treated chronically with BARD could help finding reasons for BARD-induced toxicity in humans.

Gene expression profile (GEP) analysis confirmed that BARD activated Nrf2 in kidney of ZSF1 rats. BARD consistently and specifically induced expression of gene sets downstream to Nrf2-Keap-1,

including pentose phosphate, glucuronidation, heme degradation, glutathione conjugation and eicosanoids signaling (all with FDR<1E-4; the next-best FDR was >0.05). GEP demonstrated that BARD induces potent anti-oxidant pathways. Food restriction or lisinopril, though showing some similarities in transcriptomic response, induced pathways completely divergent to those induced by BARD. In other words, the effect of BARD on GEP are with minimal overlap with those induced by either lisinopril or food restriction. Unfortunately, the major difficulty of our analysis is that the physiological effects of BARD kidney protection are concomitant with food reduction and BW loss and cannot be dissociated. It is worth noticing that BW loss induced by BARD was already reported in preclinical studies [37] [35] or in patients [38,39] [42] although without precise quantification. Therefore, this induced- BARD effect was never precisely integrated in the discussions.

The comprehensive kidney GEP analysis performed in our study has not been previously reported in other models. In short, the gene expression signatures induced by BARD suggest alternative potential protective mechanisms, not shared by food restriction or lisinopril treatment despite common physiological and metabolic (on glucose and lipids) effects.

The findings on BARD specific pathways provide a map that may guide development of new CKD therapies. Further investigation is needed to determine whether up-regulation of all or only a subset of pathways downstream to NRrf2-Keap-1 would provide the greatest efficacy with optimal safety. In particular, the mechanisms underlying potential negative cardiovascular effects, which have been observed in patients, would be of critical focus [42], [29], [64].

The gene expression analysis suggests that the most up-regulated pathways by BARD underscore its potential anti-oxidant effect. Considering the safety profile of BARD in clinical experience (mainly cardiovascular side-effects), a similar pathway-level study in cardiomyocytes and/or endothelial cells may allow differentiation of new and safer agents. In our study, the 5 most up-regulated pathways by BARD (pentose phosphate [73], glucuronidation [74], heme degradation, glutathione conjugation [75] and eicosanoids) have been associated with both chronic cardiac and renal diseases. Small

molecule screening may be performed in renal cell types, cardiomyocytes, and endothelial cells, to characterize and differentiate the gene signatures to identify safer agents acting on these pathways.

In summary, our data demonstrated that BARD protected kidney in the UNx_ZSF1 rat model, resulting in improvement of kidney function (GFR, uACR) and kidney histology (tubuli, glomeruli, basement membrane, interstitial fibrosis, inflammation, etc.). The improvement of kidney function (GFR) was reflected in reduced Alb and other BMs including KIM-1 and Cyst-C. Observed side-effects (liver findings and increased urinary MCP-1 and TGF-β1) in the UNx_ZSF1 rats treated with BARD raise the question whether BARD should be considered as a protective or a toxic treatment in general. The ZSF1 rat when combined with UNx, has features of metabolic syndrome [61] and development of CKD [60], that suggest it represents a reasonable alternative model compared to ZDF rats, DIO mice or STZ-mice. Moreover, our data -generated from two sets of ZSF1 rats- show the high reproducibility of this animal model not only after UNx was performed but also under lisinopril treatment which indicates robustness and reliability.

Conflict of Interest

None

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References

1. Martini S, Nair V, Keller BJ, Eichinger F, Hawkins JJ, et al. (2014) Integrative Biology Identifies Shared Transcriptional Networks in CKD. Journal of the American Society of Nephrology 25: 2559-2572.

2. Wild S, Roglic G, Green A, Sicree R, King H (2004) Global Prevalence of Diabetes: Estimates for the year 2000 and projections for 2030. Diabetes care 27: 1047-1053.

3. Danaei G, Finucane MM, Lu Y, Singh GM, Cowan MJ, et al. (2011) National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2•7 million participants. The Lancet 378: 31-40.

4. de Boer IH (2012) CHronic kidney disease—a challenge for all ages. JAMA 308: 2401-2402.

5. Ruggenenti P, Porrini EL, Gaspari F, Motterlini N, Cannata A, et al. (2012) Glomerular hyperfiltration and renal disease progression in type 2 diabetes. Diabetes care 35: 2061-2068.

6. Materson BJ, Preston RA (1997) Prevention of diabetic nephropathy. Hospital practice, 32: 129-134.

7. Gross JL, de Azevedo MJ, Silveiro SP, Canani LH, Caramori ML, et al. (2005) Diabetic Nephropathy: Diagnosis, Prevention, and Treatment. Diabetes Care 28: 164-176.

8. Heloisa M, Shimizu M, Coimbra TM, de Araujo M, Menezes LF, et al. (2005) N-acetylcysteine attenuates the progression of chronic renal failure. Kidney International 68: 2208-2217.

9. Cachofeiro V, Goicochea M, de Vinuesa SG, Oubina P, Lahera V, et al. (2008) Oxidative stress and inflammation, a link between chronic kidney disease and cardiovascular disease. Kidney International 74: S4-S9.

10. Del Turco S, Gaggini M, Daniele G, Basta G, Folli F, et al. (2013) Insulin resistance and endothelial dysfunction: a mutual relationship in cardiometabolic risk. Curr Pharm Des 19: 2420-2431.

11. Chow FY, Nikolic-Paterson DJ, Ozols E, Atkins RC, Tesch GH (2005) Intercellular Adhesion Molecule-1 Deficiency Is Protective against Nephropathy in Type 2 Diabetic db/db Mice. Journal of the American Society of Nephrology 16: 1711-1722.

12. Nguyen DUY, Ping FU, Mu WEI, Hill P, Atkins RC, et al. (2006) Macrophage accumulation in human progressive diabetic nephropathy. Nephrology 11: 226-231.

13. Shaker OG, Sadik NAH (2013) Transforming growth factor beta 1 and monocyte chemoattractant protein-1 as prognostic markers of diabetic nephropathy. Human & Experimental Toxicology 32: 1089-1096.

14. You H, Gao T, Cooper TK, Brian Reeves W, Awad AS (2013) Macrophages directly mediate diabetic renal injury. American Journal of Physiology - Renal Physiology 305: F1719-F1727.

15. Ruggenenti P, Cravedi P, Remuzzi G (2010) The RAAS in the pathogenesis and treatment of diabetic nephropathy. Nature reviews Nephrology 6: 319-330.

16. Santoro D, Natali A, Palombo C, Brandi LS, Piatti M, et al. (1992) Effects of chronic angiotensin converting enzyme inhibition on glucose tolerance and insulin sensitivity in essential hypertension. Hypertension 20: 181-191.

17. Zatz R, Meyer TW, Rennke HG, Brenner BM (1985) Predominance of hemodynamic rather than metabolic factors in the pathogenesis of diabetic glomerulopathy. Proceedings of the National Academy of Sciences 82: 5963-5967.

18. Brenner BM, Cooper ME, de Zeeuw D, Keane WF, Mitch WE, et al. (2001) Effects of Losartan on Renal and Cardiovascular Outcomes in Patients with Type 2 Diabetes and Nephropathy. New England Journal of Medicine 345: 861-869.

19. Terra SG (2003) Angiotensin Receptor Blockers. Circulation 107: e215-e216.

20. Appel GB, Radhakrishnan J, Avram MM, DeFronzo RA, Escobar-Jimenez F, et al. (2003) Analysis of Metabolic Parameters as Predictors of Risk in the RENAAL Study. Diabetes care 26: 1402-1407.

21. Ots M, Mackenzie HS, Troy JL, Rennke HG, Brenner BM (1998) Effects of combination therapy with enalapril and losartan on the rate of progression of renal injury in rats with 5/6 renal mass ablation. Journal of the American Society of Nephrology 9: 224-230.

22. Fried LF, Emanuele N, Zhang JH, Brophy M, Conner TA, et al. (2013) Combined Angiotensin Inhibition for the Treatment of Diabetic Nephropathy. New England Journal of Medicine 369: 1892-1903.

23. Fernandez Juarez G, Luño J, Barrio V, de Vinuesa SG, Praga M, et al. (2013) Effect of Dual Blockade of the Renin-Angiotensin System on the Progression of Type 2 Diabetic Nephropathy: A Randomized Trial. American journal of kidney diseases 61: 211-218.

24. Dong YF, Liu L, Lai ZF, Yamamoto E, Kataoka K, et al. (2010) Aliskiren enhances protective effects of valsartan against type 2 diabetic nephropathy in mice. Journal of hypertension 28: 1554-1565.

25. Sato A (2013) Mineralocorticoid receptor antagonists: their use and differentiation in Japan. Hypertension research : official journal of the Japanese Society of Hypertension 36: 185-190.

26. Ahn JH, Hong HC, Cho MJ, Kim YJ, Choi HY, et al. (2012) Effect of eplerenone, a selective aldosterone blocker, on the development of diabetic nephropathy in type 2 diabetic rats. Diabetes & metabolism journal 36: 128-135.

27. Takagi N, Tanizawa T, Kon V, Fogo AB, Ichikawa I, et al. (2012) Mineralocorticoid Receptor Blocker Protects against Podocyte-Dependent Glomerulosclerosis. Nephron Extra 2: 17-26.

28. Nishioka T, Suzuki M, Onishi K, Takakura N, Inada H, et al. (2007) Eplerenone Attenuates Myocardial Fibrosis in the Angiotensin II-Induced Hypertensive Mouse: Involvement of Tenascin-C Induced by Aldosterone-Mediated Inflammation. Journal of Cardiovascular Pharmacology 49: 261-268.

29. Rojas-Rivera J, Ortiz A, Egido J (2012) Antioxidants in kidney diseases: the impact of bardoxolone methyl. International journal of nephrology 2012: Article ID 321714, 321711 pages.

30. Zoja C, Benigni A, Remuzzi G (2013) The Nrf2 pathway in the progression of renal disease. Nephrology Dialysis Transplantation 29: i19-i24.

31. Yoh K, Itoh K, Enomoto A, Hirayama A, Yamaguchi N, et al. (2001) Nrf2-deficient female mice develop lupus-like autoimmune nephritis1. Kidney International 60: 1343-1353.

32. Aleksunes LM, Goedken MJ, Rockwell CE, Thomale J, Manautou JE, et al. (2010) Transcriptional regulation of renal cytoprotective genes by Nrf2 and its potential use as a therapeutic target to mitigate cisplatin-induced nephrotoxicity. The Journal of pharmacology and experimental therapeutics 335: 2-12.

33. Wu QQ, Wang Y, Senitko M, Meyer C, Wigley WC, et al. (2011) Bardoxolone methyl (BARD) ameliorates ischemic AKI and increases expression of protective genes Nrf2, PPARgamma, and HO-1. American journal of physiology Renal physiology 300: F1180-1192.

34. Saha PK, Reddy VT, Konopleva M, Andreeff M, Chan L (2010) The triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic-acid methyl ester has potent anti-diabetic effects in diet-induced diabetic mice and Lepr(db/db) mice. The Journal of biological chemistry 285: 40581-40592.

35. Chin M, Lee CY, Chuang JC, Bumeister R, Wigley WC, et al. (2013) Bardoxolone methyl analogs RTA 405 and dh404 are well tolerated and exhibit efficacy in rodent models of Type 2 diabetes and obesity. American journal of physiology Renal physiology 304: F1438-1446.

36. Reisman SA, Chertow GM, Hebbar S, Vaziri ND, Ward KW, et al. (2012) Bardoxolone methyl decreases megalin and activates nrf2 in the kidney. Journal of the American Society of Nephrology : JASN 23: 1663-1673.

37. Zoja C, Corna D, Nava V, Locatelli M, Abbate M, et al. (2013) Analogs of bardoxolone methyl worsen diabetic nephropathy in rats with additional adverse effects. American Journal of Physiology - Renal Physiology 304: F808-F819.

38. Pergola PE, Raskin P, Toto RD, Meyer CJ, Huff JW, et al. (2011) Bardoxolone methyl and kidney function in CKD with type 2 diabetes. The New England journal of medicine 365: 327-336.

39. Pergola PE, Krauth M, Huff JW, Ferguson DA, Ruiz S, et al. (2011) Effect of bardoxolone methyl on kidney function in patients with T2D and Stage 3b-4 CKD. American journal of nephrology 33: 469-476.

40. McCullough PA, Ali S (2012) Cardiac and renal function in patients with type 2 diabetes who have chronic kidney disease: potential effects of bardoxolone methyl. Drug design, development and therapy 6: 141-149.

41. Ruiz S, Pergola PE, Zager RA, Vaziri ND (2013) Targeting the transcription factor Nrf2 to ameliorate oxidative stress and inflammation in chronic kidney disease. Kidney Internationalernational 83: 1029-1041.

42. de Zeeuw D, Akizawa T, Audhya P, Bakris GL, Chin M, et al. (2013) Bardoxolone Methyl in Type 2 Diabetes and Stage 4 Chronic Kidney Disease. New England Journal of Medicine 369: 2492-2503.

43. Rossing P (2013) Diabetic nephropathy: Could problems with bardoxolone methyl have been predicted? Nature reviews Nephrology 9: 128–130.

44. Liu C, Chen Y, Kang Y, Ni Z, Xiu H, et al. (2011) Glucocorticoids Improve Renal Responsiveness to Atrial Natriuretic Peptide by Up-Regulating Natriuretic Peptide Receptor-A Expression in the Renal Inner Medullary Collecting Duct in Decompensated Heart Failure. Journal of Pharmacology and Experimental Therapeutics 339: 203-209.

45. Künnecke B, Verry P, Bénardeau A, von K, Markus (2004) Quantitative body composition analysis in awake mice and rats by magnetic resonance relaxometry. Obesity research 12: 1604-1615.

46. Qi Z, Breyer MD (2009) Measurement of glomerular filtration rate in conscious mice. Methods Mol Biol 466: 61-72.

47. Lorenz JN, Gruenstein E (1999) A simple, nonradioactive method for evaluating single-nephron filtration rate using FITC-inulin. American Journal of Physiology - Renal Physiology 276: F172-F177.

48. Gautier L, Cope L, Bolstad BM, Irizarry RA (2004) affy—analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 20: 307-315.

49. Smyth GK (2005) Limma: linear models for microarray data. Bioinformatics and computational biology solutions using R and Bioconductor: Springer. pp. 397-420.

50. Sturgeon C, Sam AD, 2nd, Law WR (1998) Rapid determination of glomerular filtration rate by single-bolus inulin: a comparison of estimation analyses. Journal of applied physiology 84: 2154-2162.

51. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, et al. (2005) Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. Proceedings Of The National Academy Of Sciences Of The United States Of America 102: 15545-15550.

52. Wu D, Smyth GK (2012) Camera: a competitive gene set test accounting for inter-gene correlation. Nucleic acids research 40: e133.

53. Matthews L, Gopinath G, Gillespie M, Caudy M, Croft D, et al. (2009) Reactome knowledgebase of human biological pathways and processes. Nucleic acids research 37: D619-D622.

54. Schaefer CF, Anthony K, Krupa S, Buchoff J, Day M, et al. (2009) PID: the pathway interaction database. Nucleic acids research 37: D674-D679.

55. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B (Methodological): 289-300.

56. Hong DS, Kurzrock R, Supko JG, He X, Naing A, et al. (2012) A Phase I First-in-Human Trial of Bardoxolone Methyl in Patients with Advanced Solid Tumors and Lymphomas. Clinical Cancer Research 18: 3396-3406.

57. Bilan VP, Salah EM, Bastacky S, Jones HB, Mayers RM, et al. (2011) Diabetic nephropathy and long-term treatment effects of rosiglitazone and enalapril in obese ZSF1 rats. J Endocrinol 210: 293-308.

58. Benardeau A, Riboulet W, Albertini M, Gomez M (2013) Animal models of metabolic syndrome and associated co-morbidities. Journal of Experimental and Applied Animal Science 1: 152-155.

59. Soler MJ, Riera M, Batlle D (2012) New experimental models of diabetic nephropathy in mice models of type 2 diabetes: efforts to replicate human nephropathy. Experimental diabetes research 2012: 1-9.

60. Tofovic SP KH, Kost CK Jr, Bastacky S (2000) Renal function and structure in diabetic, hypertensive, obese ZDFxSHHF-hybrid rats. Renal failure 22: 387-406.

61. Tofovic SP, Jackson EK (2003) Rat Models of the Metabolic Syndrome. Renal Disease -Methods in Molecular Medicine-. pp. 29-46.

62. Griffin KA, Abu-Naser M, Abu-Amarah I, Picken M, Williamson GA, et al. (2007) Dynamic blood pressure load and nephropathy in the ZSF1 (fa/facp) model of type 2 diabetes. American Journal of Physiology - Renal Physiology 293: F1605-F1613.

63. Brenner BM, Lawler EV, Mackenzie HS (1996) The hyperfiltration theory: A paradigm shift in nephrology. Kidney International 49: 1774-1777.

64. Thomas M (2012) A preliminary evaluation of bardoxolone methyl for the treatment of diabetic nephropathy. Expert Opinion on Drug Metabolism & Toxicology 8: 1015-1022.

65. Ritz E (2013) Bariatric surgery and the kidney—much benefit, but also potential harm. Clinical Kidney Journal 6: 368-372.

66. Neff KJ, Frankel AH, Tam FWK, Sadlier DM, Godson C, et al. (2013) The effect of bariatric surgery on renal function and disease: a focus on outcomes and inflammation. Nephrology Dialysis Transplantation 28: iv73-iv82.

67. Tirosh A, Golan R, Harman-Boehm I, Henkin Y, Schwarzfuchs D, et al. (2013) Renal Function Following Three Distinct Weight Loss Dietary Strategies During 2 Years of a Randomized Controlled Trial. Diabetes care 36: 2225-2232.

68. Lock EA (2010) Sensitive and early markers of renal injury: where are we and what is the way forward? Toxicol Sci 116: 1-4.

69. Bonventre JV, Vaidya VS, Schmouder R, Feig P, Dieterle F (2010) Next-generation biomarkers for detecting kidney toxicity. Nature biotechnology 28: 436-440.

70. Fuchs TC, Hewitt P (2011) Biomarkers for drug-induced renal damage and nephrotoxicity-an overview for applied toxicology. The AAPS Journal 13: 615-631.

71. Wang C, Li C, Gong W, Lou T (2013) New urinary biomarkers for diabetic kidney disease. Biomarker Research 1: 1-4.

72. Thomas MC (2011) Bardoxolone: Augmenting the Yin in chronic kidney disease. Diabetes and Vascular Disease Research 8: 303-304.

73. Katare R, Oikawa A, Cesselli D, Beltrami AP, Avolio E, et al. (2013) Boosting the pentose phosphate pathway restores cardiac progenitor cell availability in diabetes. Cardiovascular research 97: 55-65.

74. Xu Y, Zhang Z, Hu J, Stillman IE, Leopold JA, et al. (2010) Glucose-6-phosphate dehydrogenase-deficient mice have increased renal oxidative stress and increased albuminuria. The FASEB Journal 24: 609-616.

75. Annuk M, Zilmer M, Lind L, Linde T, Fellström B (2001) Oxidative Stress and Endothelial Function in Chronic Renal Failure. Journal of the American Society of Nephrology 12: 2747-2752.

76. Culhane A, Perriere G, Higgins D (2003) Cross-platform comparison and visualisation of gene expression data using co-inertia analysis. BMC Bioinformatics 4: 59.

Supplemental web links

http://www.mayoclinic.org/drugs-supplements/lisinopril-oral-route/precautions/drg-20069129

http://www.livestrong.com/article/494553-the-side-effects-of-lisinopril-on-glucose

www.drugs.com/cdi/lisinopril.html

http://doublecheckmd.com/EffectsDetail.do?dname=lisinopril-hydrochlorothiazide&sid=19460&eid=2222)

http://www.drugs.com/sfx/hydrochlorothiazide-lisinopril-side-effects.html

Figures and tables

Figure 1 **Body Weight (A), Food Intake (B), averaged Food Intake per week (C) and diuresis (D) measured in ZSF1 rats receiving vehicle, Lisinopril (30 mg/kg), BARD (10 mg/kg). Values are expressed as mean ± SEM. ***P < 0.001 vs. vehicle.**

Figure 2 **Mean arterial pressure (MAP) in ZSF1 rats treated with vehicle, Lisinopril (30 mg/kg), BARD (10 mg/kg). Values are expressed as mean ± SEM. ***P < 0.001 vs. vehicle.**

Figure 3 **Plasma creatinine (A) and plasma KIM-1 (B) levels measured in ZSF1 rats after completion of treatment with vehicle, Lisinopril (30 mg/kg), BARD (10 mg/kg). Values are expressed as mean ± SEM. ***P < 0.001 vs. vehicle.**

Figure 4 **Liver of ZSF1 rats treated with (A) vehicle demonstrating hepatocellular vacuolation and rarefaction, and (B) BARD with evidence of bile duct hyperplasia. Hematoxylin-eosin staining (HE).**

Figure 5 **Glomerular Filtration Rate (GFR) measured (A) and urinary albumin-creatinine ratio (uACR) calculated (B) in ZSF1 rats treated with vehicle, Lisinopril (30 mg/kg), BARD (10 mg/kg). Values are expressed as mean ± SEM. ***P < 0.001 vs. vehicle.**

Figure 6 **Urinary markers of kidney function; (A) Albumin, (B) KIM-1, (C) Cystatin-C and (D) β2-microglobulin daily excretion in ZSF1 rats treated with vehicle, Lisinopril (30 mg/kg), BARD (10 mg/kg). Values are expressed as mean ± SEM. *P < 0.05; ***P < 0.001 vs. vehicle.**

Figure 7 **Urinary markers of inflammation: (A) TGF-β1 and (B) MCP-1 urine daily excretion in ZSF1 rats treated with vehicle, Lisinopril (30 mg/kg) or BARD (10 mg/kg). Values are expressed as mean ± SEM. *P< 0.05; ***P < 0.001 vs. vehicle.**

Figure 8 **Kidney mean glomerulosclerosis score (GS) evaluated in ZSF1 rats treated with vehicle, Lisinopril (30 mg/kg), BARD (10 mg/kg).**

Figure 9 **Pathological scoring of renal findings in ZSF1 rats treated with vehicle, Lisinopril (30 mg/kg), BARD (10 mg/kg). Values are expressed as mean ± maximum and minimum findings.**

Figure 10 **Histopathology of kidneys of ZSF1 rats treated with (A) vehicle, (B) BARD and (C) Lisinopril or (D) under food restriction. Basement membrane thickening (arrow) and glomerulosclerosis (star); PAS stain.**

Figure 11 **Infiltration of CD68 positive cells (macrophages, A-D) in kidneys of ZSF1 rats treated with (A) vehicle, (B) BARD (10mg/kg) (C) Lisinopril (30 mg/kg) or (D) under food restriction. Immunohistochemistry was performed with an antibody directed against CD68, DAB stain.**
Infiltration of CD3 positive cells (lymphocytes, E-H) in kidneys of ZSF1 rats treated with (E) Vehicle, (F) BARD (10mg/kg), (G) Lisinopril (30 mg/kg) or (D) food restriction (H). Immunohistochemistry was performed with an Antibody directed against CD3, DAB staining.

Figure 12 **Body composition measured in the Vehicle and food restriction groups between 3 and 7 weeks of treatment. Values are expressed as mean ± SEM.**

Figure 13 **Whole genome mRNA expression profiles of four treatment groups revealed by the multivariate co-inertia analysis [76]. The first (x) and second (y) axis captures 24.8% and 10.8% of total variability, respectively. Samples are colored by treatment group and their distributions are illustrated by inertia ellipses. Between-group changes are observed primarily in the first axis, suggesting that Lisinopril, food restriction, and BARD all induce global gene expression changes and their effects share some similarity. At the same time, BARD's effect is different from food restriction (primarily on the second axis). This suggests Bardoxolone's effect cannot be completely explained by food restriction (see main text).**

Figure 14 **Significant up-regulation of several biological networks downstream of the Keap1-Nrf2 pathway. We tested for systematic up- or down-regulation of a comprehensive list of 1396 biological networks using competitive gene set test methods GSEA and CAMERA. The following pathways were positively regulated by BARD (FDR<1E-4): (A) pentose phosphate (PPP), (B) glucuronidation, (C) heme degradation and (D) glutathione conjugation pathways. They are all established targets of the Keap1-Nrf2 pathway. To further validate this finding, we also performed gene set analysis with transcription factor target genes derived from literature. Nrf2-target genes were found significantly up-regulated (FDR<1E-4, data not shown).**

Figure 15 **A schematic showing the main 4-5 downstream pathways specifically up-regulated by BARD.**

Figure 16 **(A) Body Weight, (B) Food Intake, (C) MAP and (D) GFR measured in ZSF1 rats treated with vehicle, Lisinopril (30 mg/kg), under food restriction. Values are expressed as mean ± SEM. ***P<0.001 vs. vehicle.**

Figure 17 **Urinary markers of kidney function; (A) albumin, (B) KIM-1, (C) Cystatin-C, (D) β2-microglobuline , (E)TGF-β1 and (F) MCP-1 daily excretion in ZSF1 rats treated with vehicle, Lisinopril (30 mg/kg), and under food restriction. Values are expressed as mean ± SEM. *P < 0.05; ***P < 0.001 vs. vehicle.**

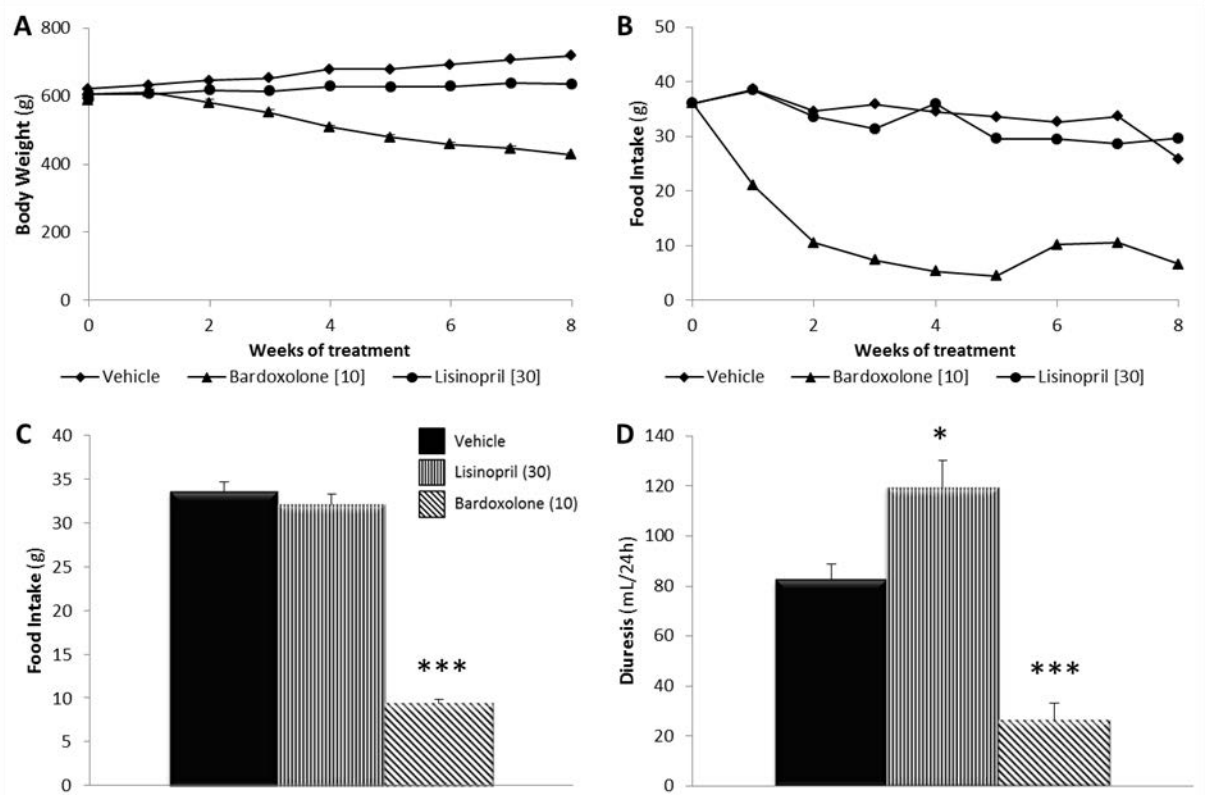
Figure 18 **Kidney mean differential glomerulosclerosis score (GS) evaluated in ZSF1 rats treated with vehicle, Lisinopril (30 mg/kg), or under food restriction.**

Table 1 **Plasma metabolic parameters in Study 1 measured at the end of the treatment. Values are expressed as mean ± SEM. ***P < 0.001 vs. vehicle.**

Table 2 **Organ weights as a % of body weight evaluated at the end of the treatment. Values are expressed as mean ± SEM. **P < 0.01; ***P < 0.001 vs. vehicle.**

Table 3 **Organ weights as absolute values evaluated at the end of the treatment. Values are expressed as mean ± SEM. **P < 0.01; ***P < 0.001 vs. vehicle.**

Table 4 **Plasma metabolic parameters in Study 2 measured at the end of the treatment in vehicle, Lisinopril (30 mg/kg) and food restricted group. Values are expressed mean ± SEM. *P<0.05, **P<0.01 and ***P < 0.001 vs. vehicle.**



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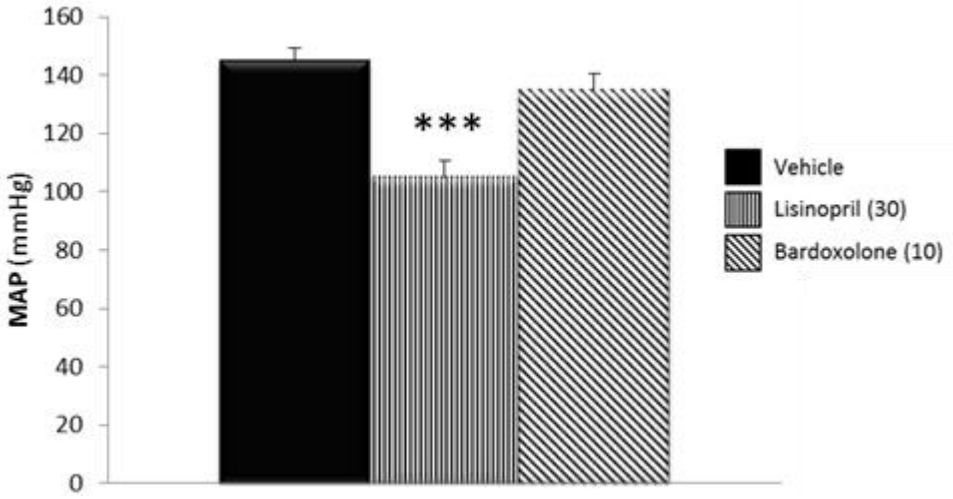

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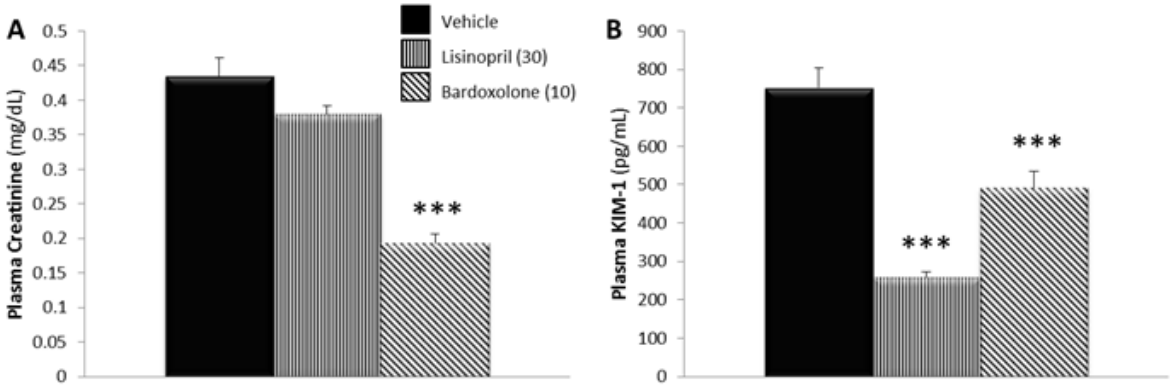

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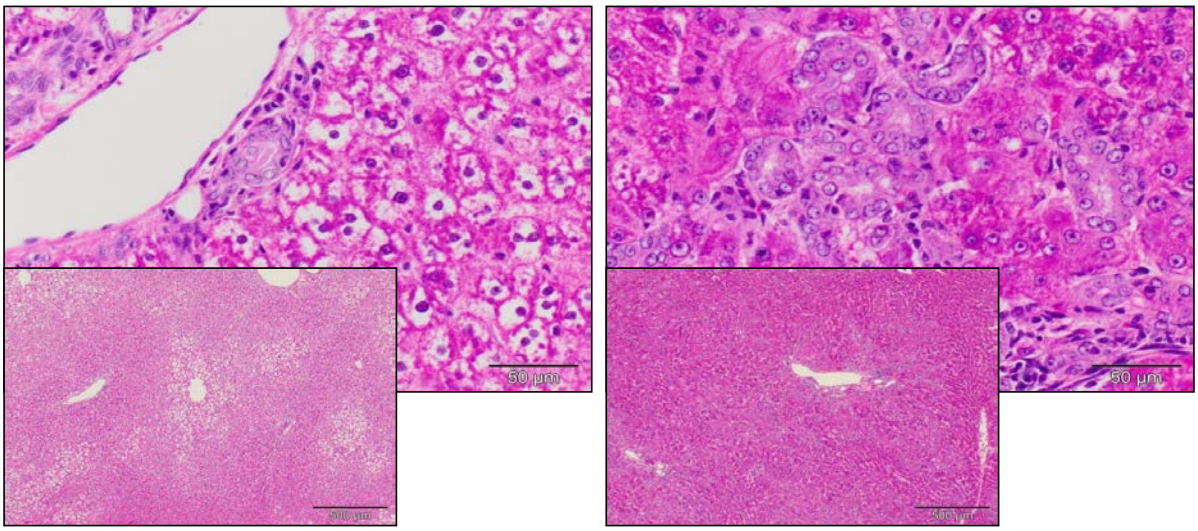
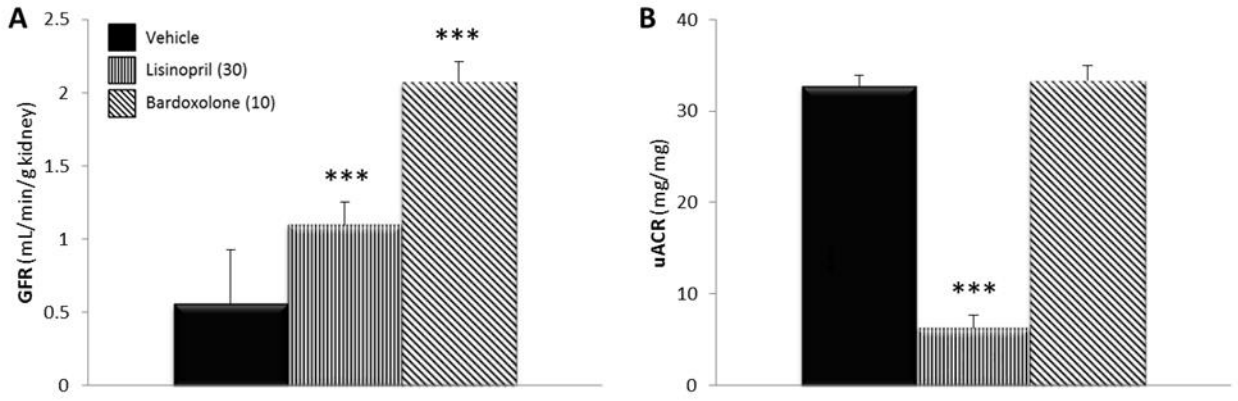


A. Liver (vehicle), vacuolation, HE

B. Liver (5), bile duct hyperplasia, HE

Figure 4



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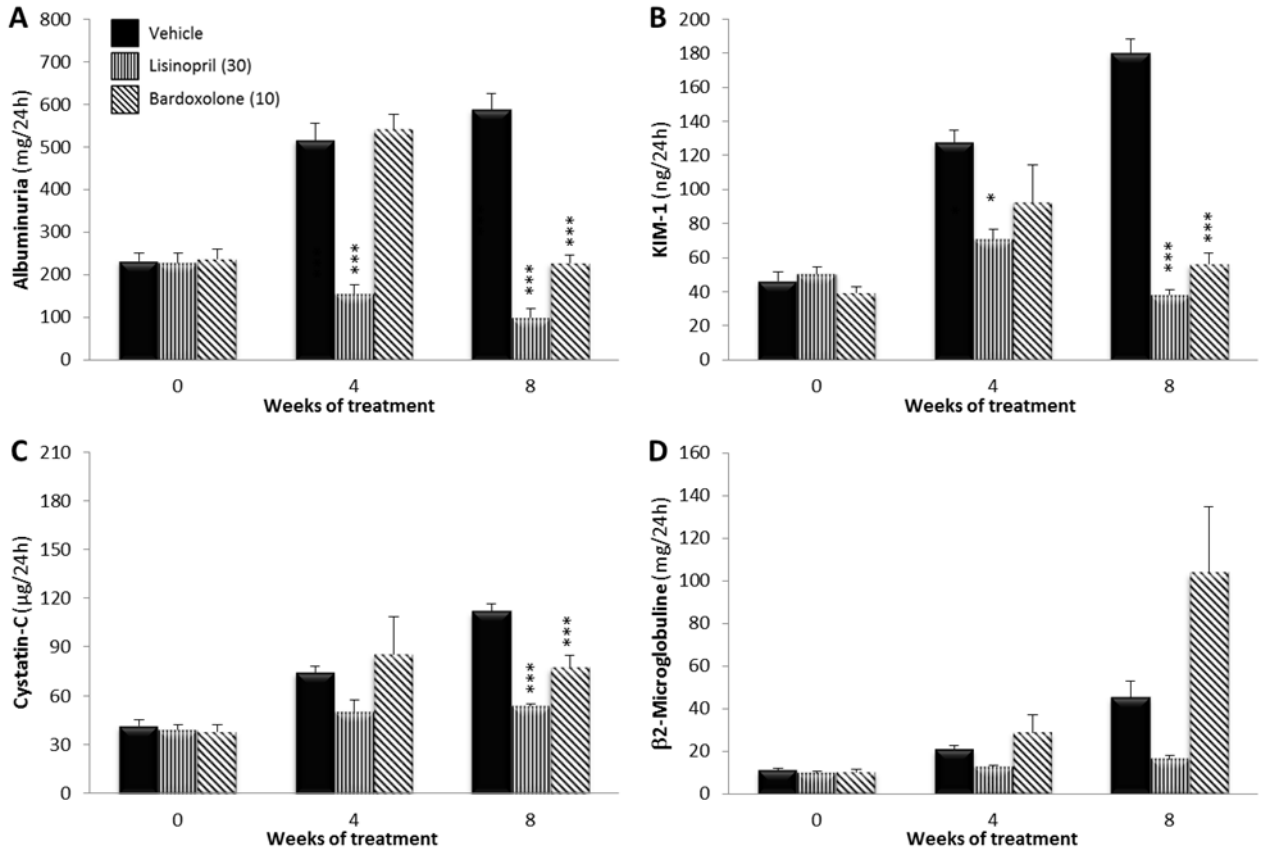

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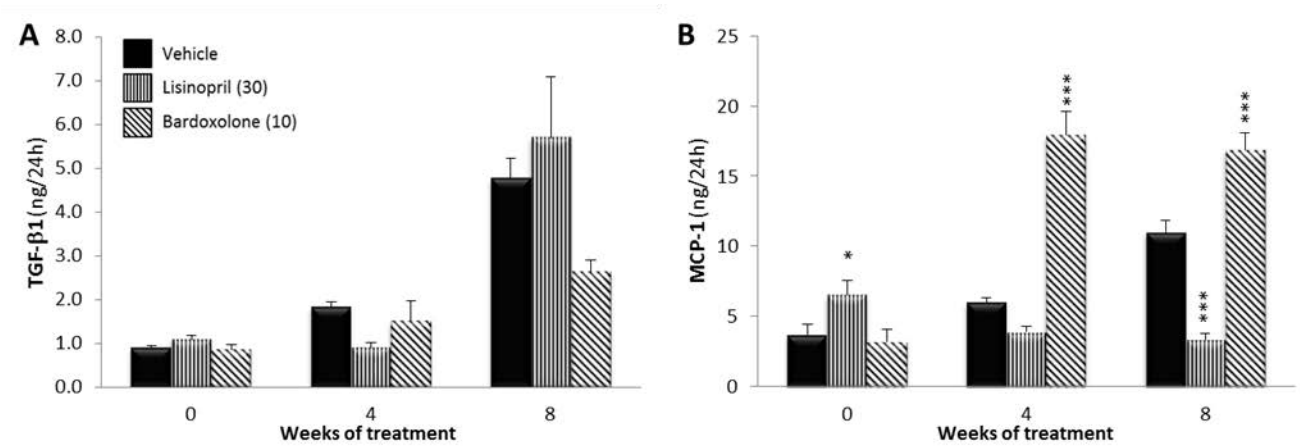

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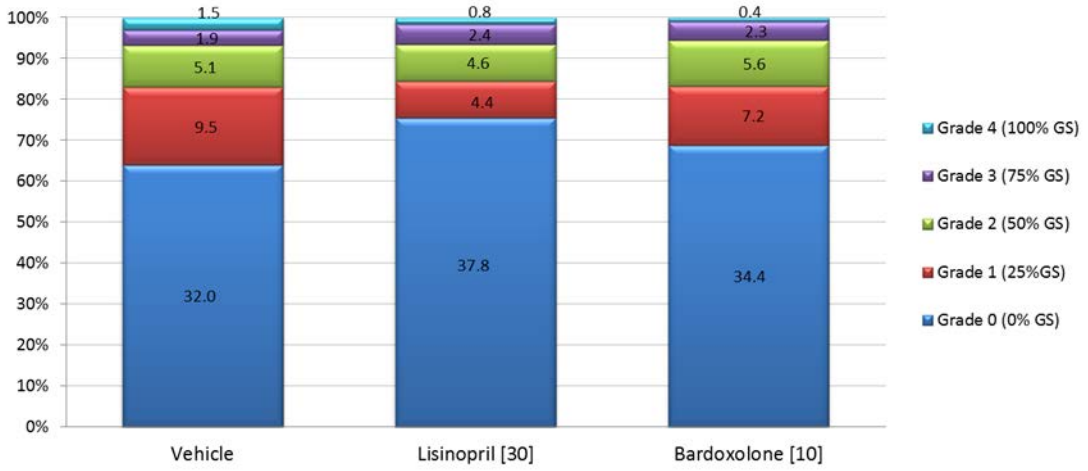

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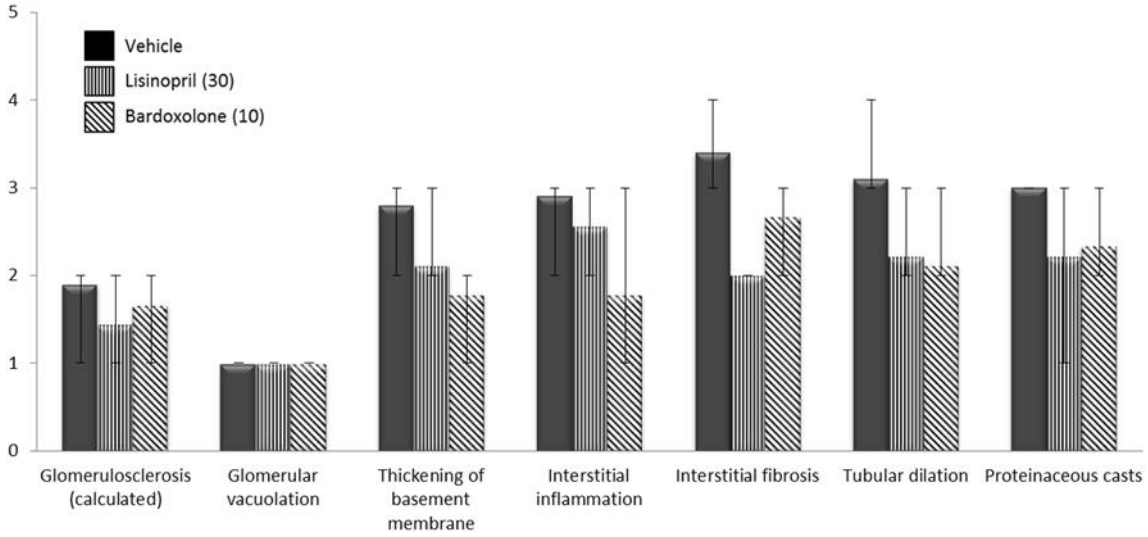

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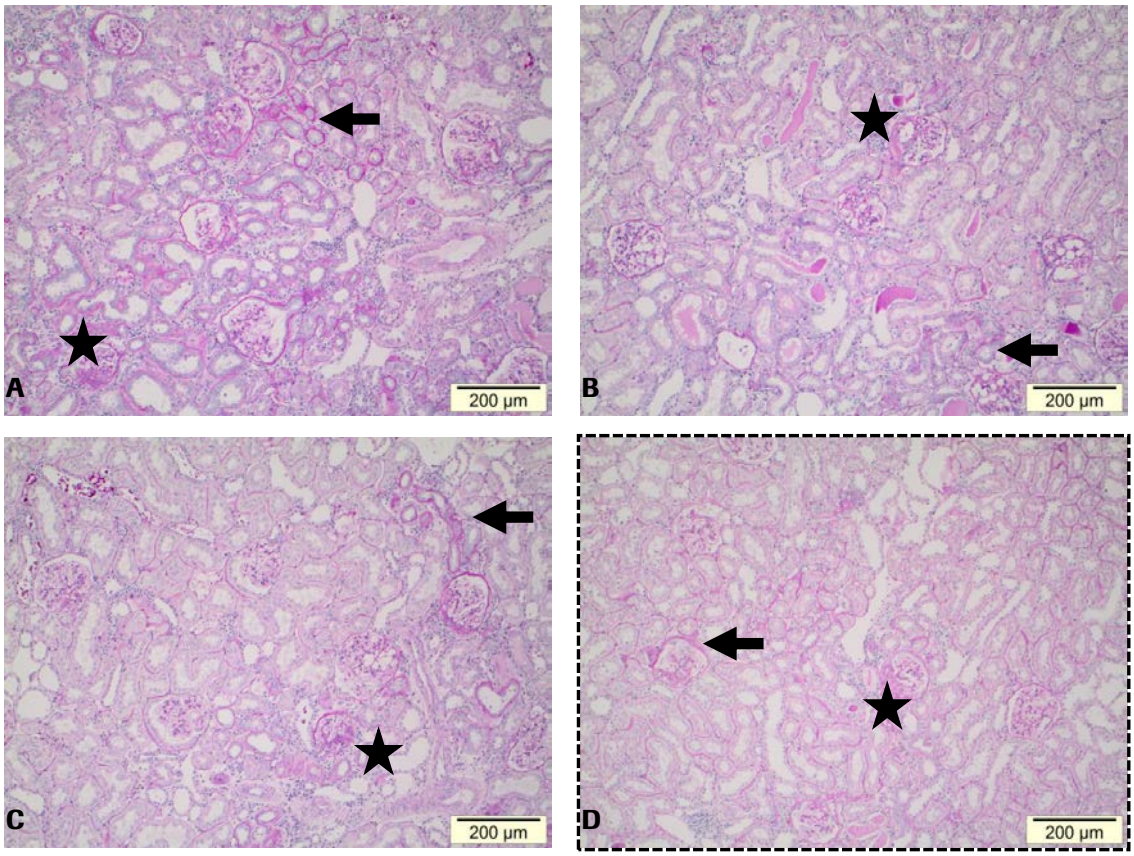

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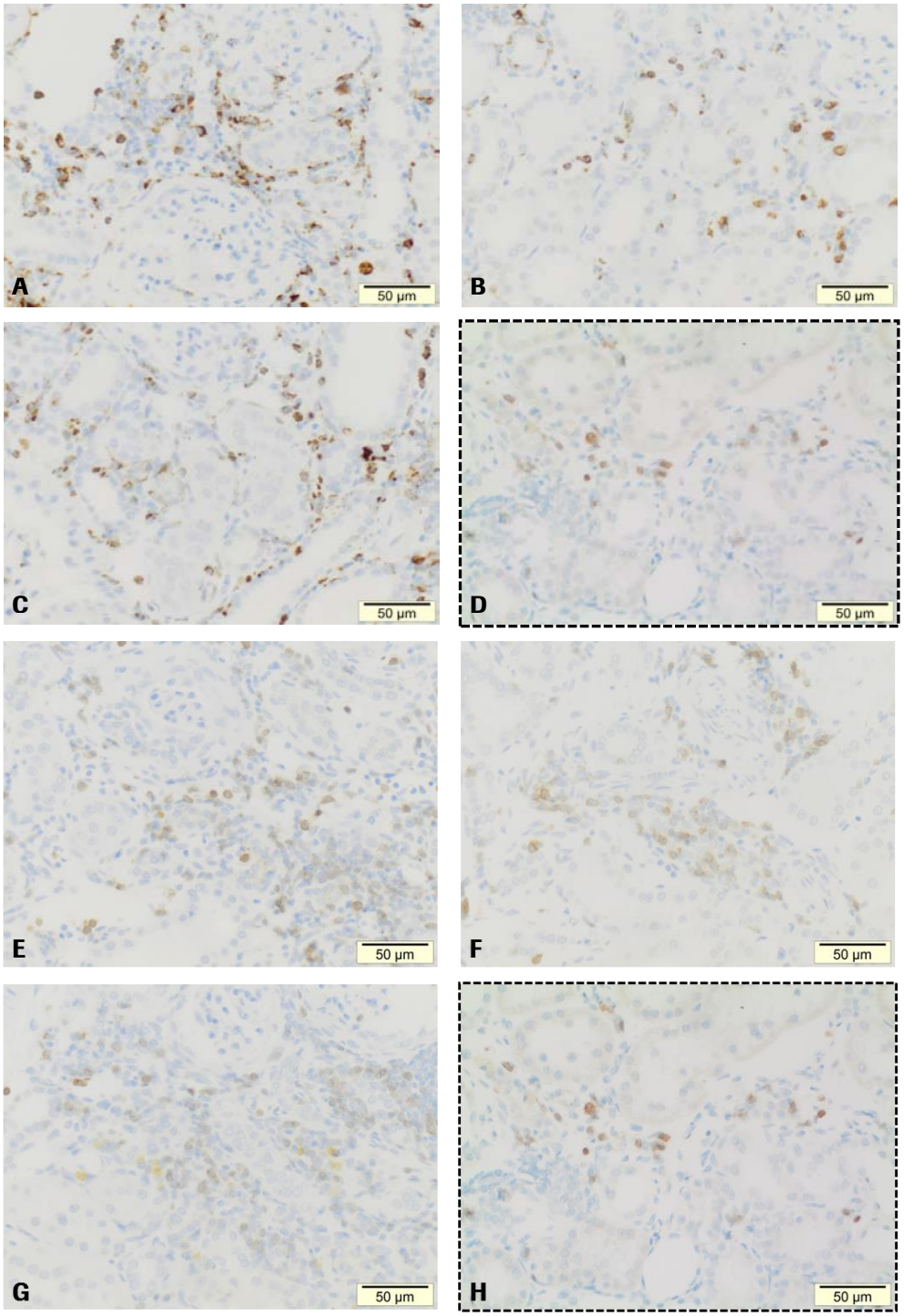

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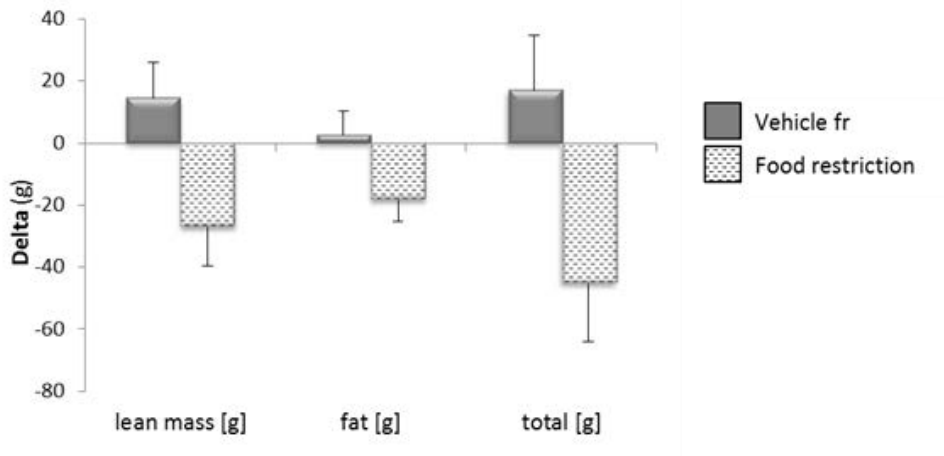

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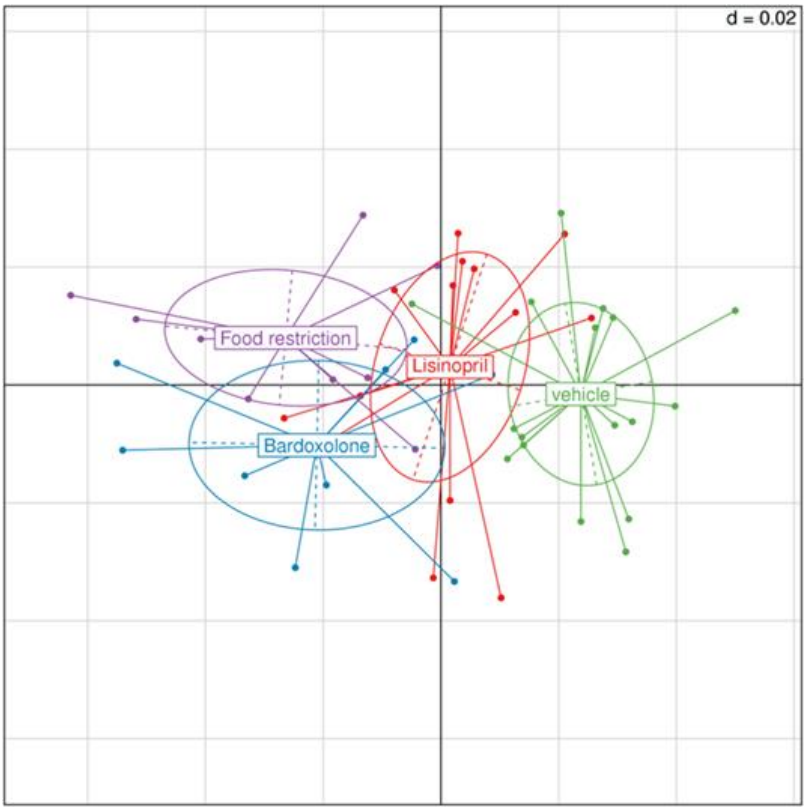

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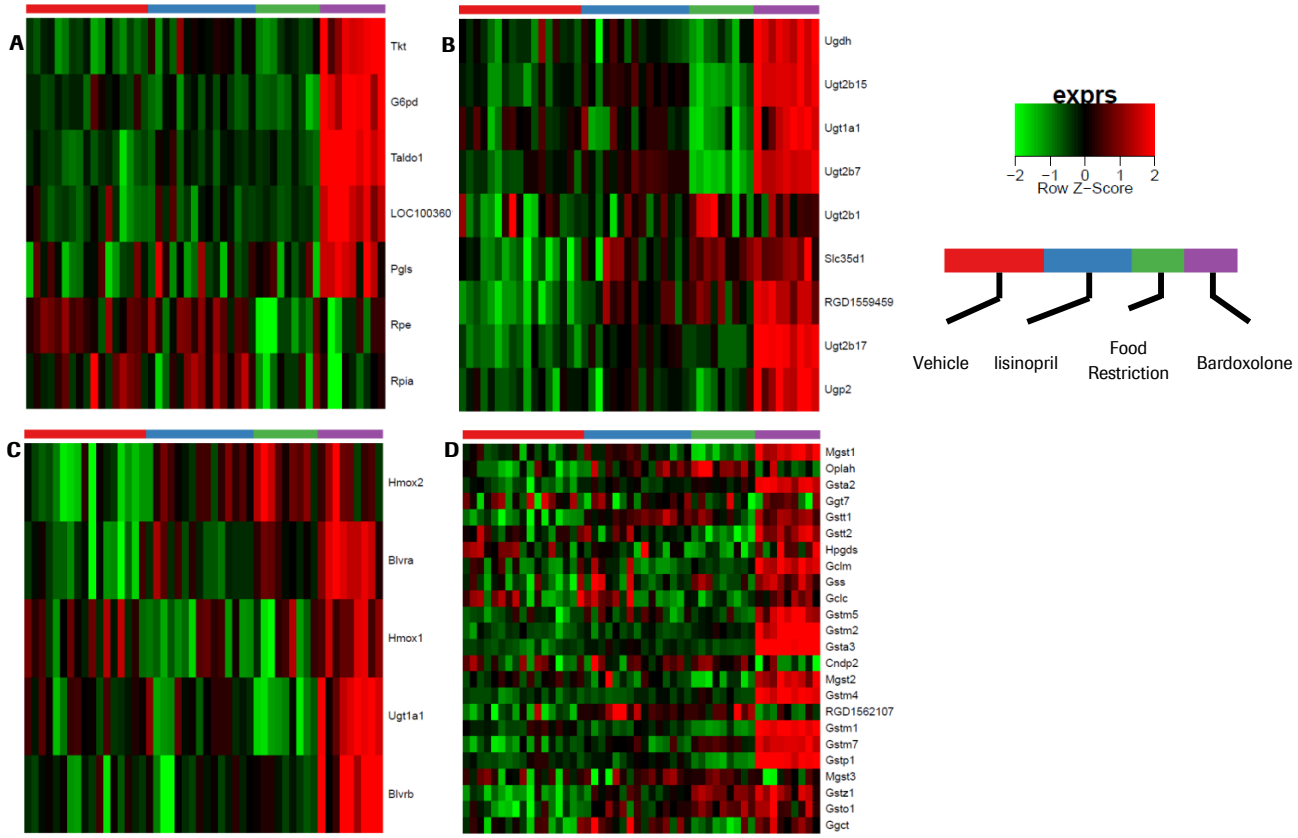

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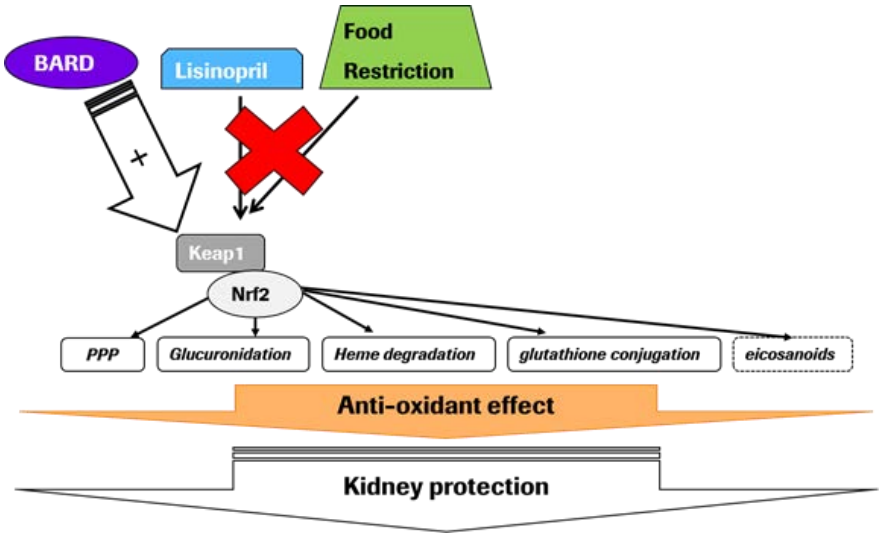

Figure 15

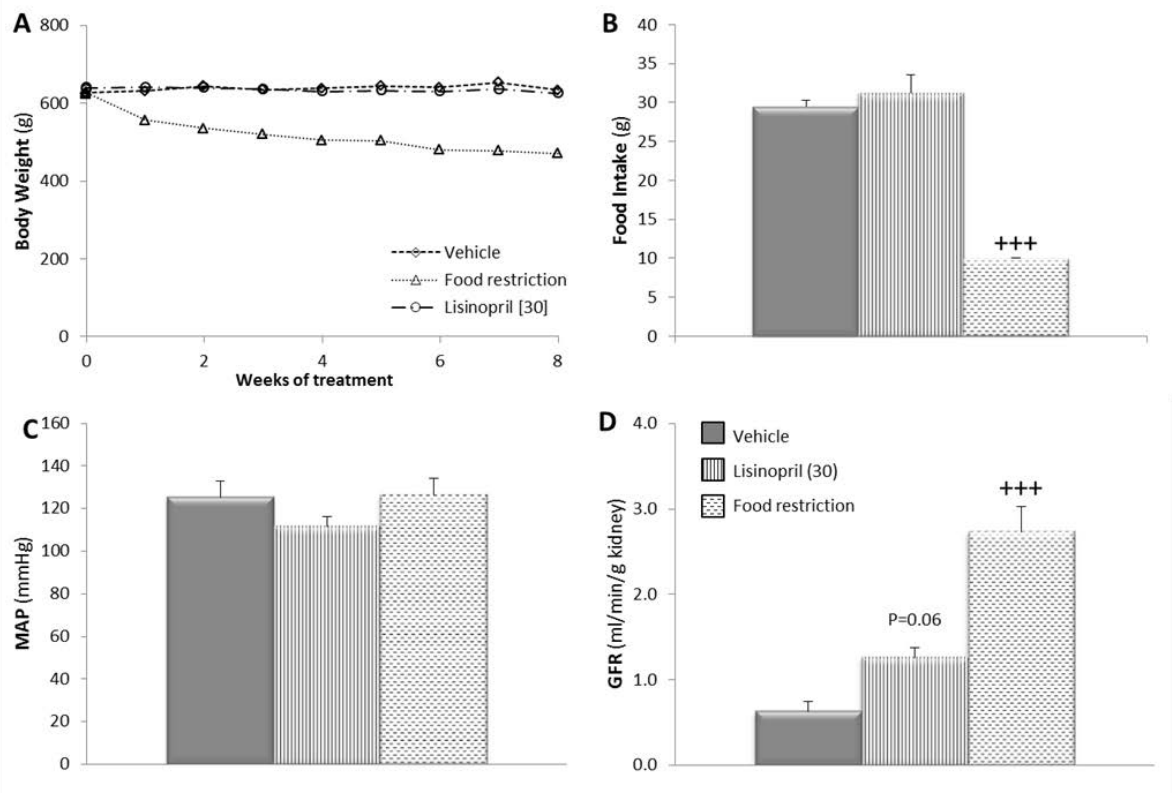
A
Body Weight (g)
Weeks of treatment
Vehicle
Food restriction
Lisinopril [30]
B
Food Intake (g)
+++
C
MAP (mmHg)
D
GFR (ml/min/g kidney)
Vehicle
Lisinopril (30)
Food restriction
+++
P=0.06

Figure 16

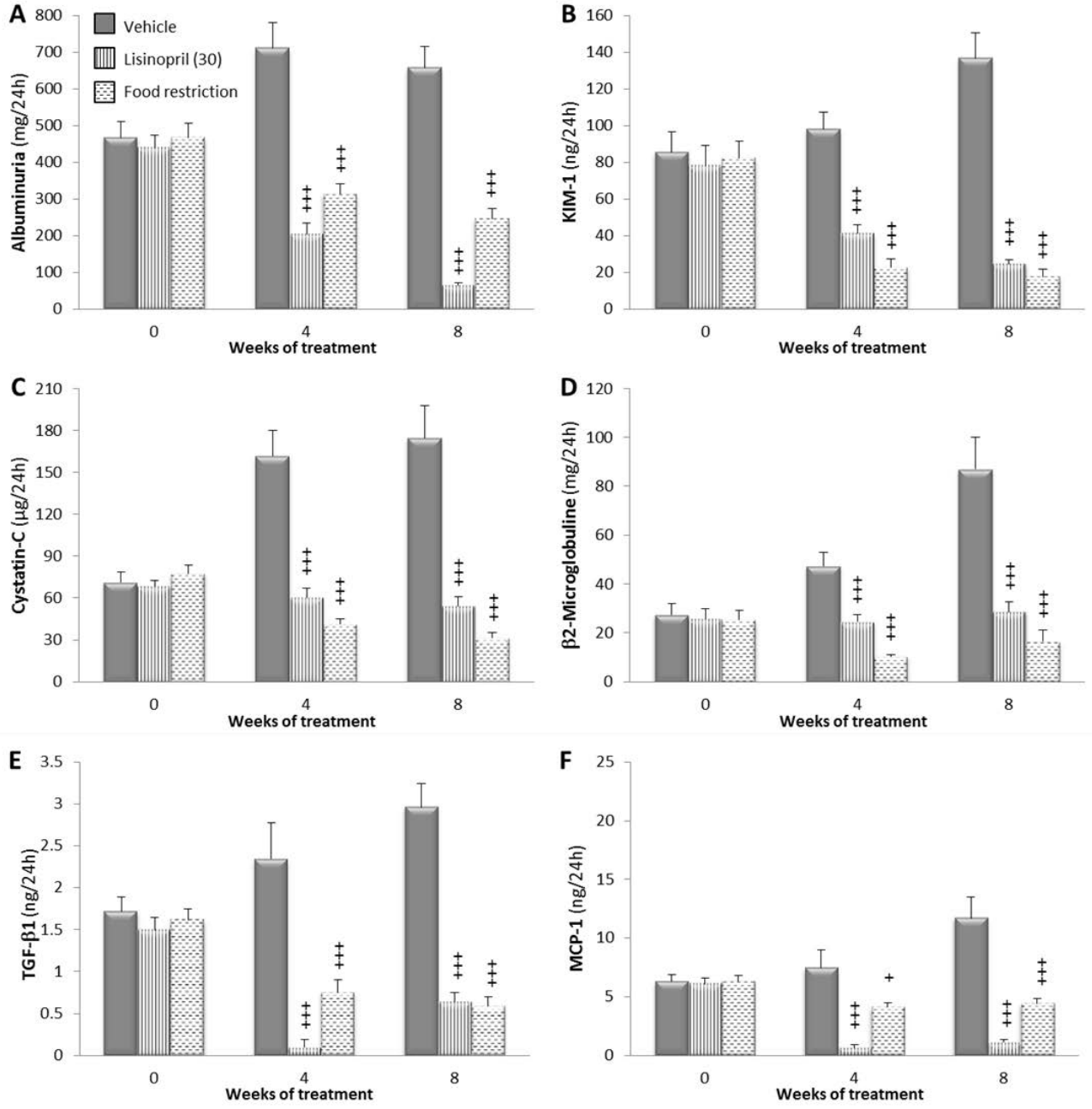
A
Vehicle
Lisinopril (30)
Food restriction
Albuminuria (mg/24h)
Weeks of treatment
B
KIM-1 (ng/24h)
Weeks of treatment
C
Cystatin-C (µg/24h)
Weeks of treatment
D
β2-Microglobuline (mg/24h)
Weeks of treatment
E
TGF-β1 (ng/24h)
Weeks of treatment
F
MCP-1 (ng/24h)
Weeks of treatment

Figure 17

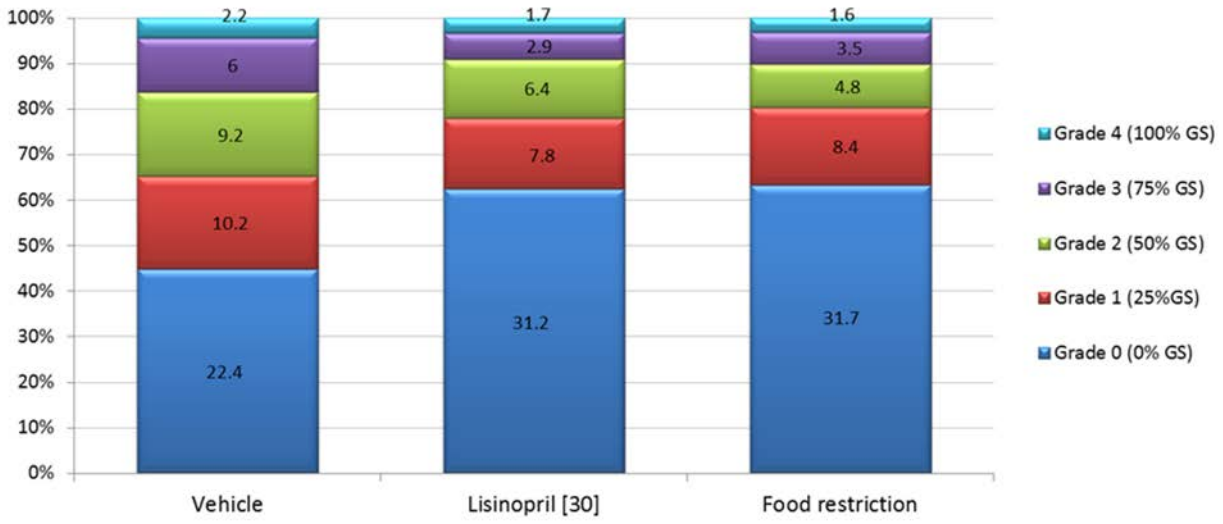

Figure 18

Table 1

Plasma biomarkers	Vehicle	Lisinopril	Bardoxolone
Glucose (fed) (mmol/l)	24 (2.5)	36.3 (1.3) ***	8.1 (0.5) ***
Insulin (fed) (ng/ml)	9.32 (1.69)	8.06 (1.21)	0.53 (0.04)
Triglycerides (mmol/l)	78.9 (7.3)	38.6 (4.2) ***	4.7 (0.4) ***
NEFA (mmol/l)	1.54 (0.21)	0.73 (0.08) ***	0.24 (0.04) ***
Total cholesterol (mmol/l)	17.4 (1.0)	9.9 (0.8) ***	15.2 (0.9)

Table 2

Organs weight	Vehicle	Lisinopril	Bardoxolone
Kidney (g/100g Body weight)	0.59 (0.02)	0.53 (0.02)	0.73 (0.03) ***
Liver (g/100g Body weight)	6.37 (0.14)	5.68 (0.17)	16.48 (0.76) ***
Heart (g/100g Body weight)	0.25 (0.01)	0.23 (0.00)**	0.32 (0.01) ***
Epi-Fat (g/100g Body weight)	1.94 (0.09)	1.36 (0.04)	1.98 (0.08) ***

Table 3

Organs weight	Vehicle	Lisinopril	Bardoxolone
Kidney (g)	4.2 (0.1)	3.3 (0.1) ***	3.0 (0.1) ***
Liver (g)	45.7 (1.6)	35.7 (2.1)	68.1 (3.3) **
Heart (g)	1.81 (0.04)	1.42 (0.05) ***	1.33 (0.02) ***
Epi-Fat (g)	14.0 (0.9)	12.3 (0.6)	5.6 (0.2) ***

Table 4

Plasma biomarkers	Vehicle Study 2	Lisinopril Study 2	Food restriction Study 2
Glucose (fed) (mmol/l)	20.2 (1.5)	25.2 (1.8) *	14.2 (0.9) **
Insulin (fed) (ng/ml)	6.0 (0.4)	6.6 (0.8)	3.6 (0.5) *
Triglycerides (mmol/l)	26.4 (6.3)	32.8 (3.4)	14.1 (0.9)
NEFA (mmol/l)	0.87 (0.11)	0.45 (0.03) ***	0.22 (0.01) ***
Total cholesterol (mmol/l)	15.3 (1.2)	8.3 (0.5) ***	8.4 (0.3) ***

Discussion - Conclusion

Comme attendu, le lisinopril a protégé la fonction rénale, et réduit la pression artérielle chez le rat UNx-ZSF1. Le bardoxolone a protégé la fonction rénale dans une mesure comparable au lisinopril, a montré une amélioration des biomarqueurs mesurés, et normalisé les paramètres métaboliques sans avoir d'impact sur la pression artérielle. De plus, le bardoxolone a mis en évidence une baisse de la prise alimentaire de ces animaux hyperphagiques ainsi qu'une baisse de poids certainement associée. Ces effets bénéfiques du bardoxolone sont certainement en partie médiés par l'amélioration du statut métabolique de ces animaux, à savoir notamment par la perte de poids. Les deux traitements bardoxolone et lisinopril ont montré des effets protecteurs similaires sur les structures glomérulaire et tubulaire. Les profils d'expression génique ont montré des régulations différentes selon le traitement: les gènes régulés par Nrf2 ont été surexprimés par le bardoxolone seulement et non au sein du groupe soumis à une restriction alimentaire. Par conséquent, nos données suggèrent que les effets protecteurs du bardoxolone sont en partie dus à l'amélioration générale du statut métabolique des rats UNx-ZSF (perte de poids induite), et par l'activation de la voie Nrf-2, indépendamment de la pression sanguine.

Discussion - Conclusion

As expected, lisinopril improved kidney function and reduced blood pressure of UNx-ZSF1 rats. Bardoxolone improved kidney function, kidney biomarkers and normalized metabolic parameters without changing blood pressure. Bardoxolone concomitantly and significantly reduced body weight and food intake. Our data indicate that bardoxolone can protect kidney in a same extent as lisinopril does in this rat model of metabolic syndrome and diabetic nephropathy. Both treatments similarly decreased glomerular and tubular damage as determined by histological score. Effects of bardoxolone suggested that improvement of metabolic status due to body weight reduction could contribute to improvement of kidney function. Gene expression profile indicated mainly different

pathways regulation between lisinopril, bardoxolone and food restriction group. Nrf2-regulated pathways were significantly up-regulated by bardoxolone, while neither lisinopril nor food restriction showed specific pathway-level regulation.

Our data suggest that the effects of bardoxolone in ZSF1 are driven partially by body weight loss and partially by induction of Nrf2-responsive genes, and are independent of blood pressure reduction. Liver side-effects induced by bardoxolone and similarly observed in patients, suggesting that ZSF1 is becoming an attractive model for investigating kidney progression and sensitivity of new treatment in a context of metabolic syndrome.

To conclude on the UNx methodology to worsen the ZSF1 model, we demonstrated that:

- Kidney structure and function were worsened by UNx in terms of extent and onset as compared to non-UNx ZSF1 rats.
- This UNx-ZSF1 rat model is suitable for testing standard of care and is sensitive to innovative compounds acting on renal structure and function, in a context of metabolic syndrome.

Nevertheless, we wished to exacerbate both renal <u>and</u> cardiac defects in terms of extent of the structural damages and functional decreases. The final aim was to improve the ZSF1 rat model of metabolic syndrome for being able to test potential protective effects of new compounds on both renal and cardiac function in a reasonable time frame (less than a year). As a result, our first tentative with the UNx was a semi-success as blood pressure, and related cardiac structure and function were not altered by this surgical approach, contrary to what we could have expected (see annex 1).

Our second strategy was to induce a hormonal stress with the infusion of Angiotensin II for 6 weeks. With this strategy, we aimed at shortening the onset of renal defects to trigger simultaneously cardiac alterations.

2.6 ANGIOTENSIN II INFUSION IN THE ZSF1 RAT MODEL AS A STRATEGY TO DETERIORATE CARDIAC AND RENAL FUNCTIONS

As evoked in Section 1.5.1, AngII is a potent hypertensive hormone that promotes inflammation, ROS formation, interstitial fibrosis and hypertrophy and leads to aldosterone release. Moreover, as mentioned in Section 1.5.3, and as displayed in Figure 11, aldosterone, *via* mineralocorticoid receptor signaling, negatively impacts heart and kidney. In kidney, aldosterone is involved in glomerulosclerosis, tubulointerstitial inflammation and fibrosis, oxidative stress, podocyte dysfunction and proteinuria. In the heart, it is related to cardiovascular inflammation, fibrosis and hypertrophic remodeling (Briet and Schiffrin 2011).

Based on the above statements, we hypothesized that infusing AngII could induce dual effects:

- an hemodynamic stress, by blood pressure increase leading to nephron defects (Bidani and Griffin 2004), on top of increasing fibrosis, oxidative stress and inflammation,
- a hormonal stress, induced by the increase in aldosterone release due to the artificial RAAS enhancement, also related to inflammation, fibrosis, and oxidative stress.

We thought that these 2 main effects could lead to exacerbation of both renal and cardiac structures and functions impairment in terms of extent and onset, and lead to a cardio-renal disease. Would these conditions lead to a cardio-renal model, AngII-infused ZSF1 rat could become a model for testing aldosterone synthase inhibitor on cardiac and renal structures and functions.

PUBLICATION n° 4

ALDOSTERONE SYNTHASE INHIBITOR REVERSES KIDNEY DAMAGE IN A NOVEL DIABETIC AND CARDIO-RENAL RAT MODEL

Introduction

Cette étude s'est déroulée en deux parties. Dans la première nous avons tenté d'exacerber les dysfonctions rénale et cardiaque du modèle ZSF1 développant un syndrome métabolique, afin que ces dysfonctions se développent en un temps plus court que sans intervention, et avec une sévérité plus importante. Pour cela, nous avons induit un stress hémodynamique et hormonal par l'infusion d'AngII durant 6 semaines, le but étant d'induire une forte hypertension, ainsi qu'une surproduction d'aldostérone. De ce fait nous espérions accélérer et développer les lésions engendrées au niveau du rein et du cœur. De plus nous avons ajouté la mesure du Growth Differentiation Factor-15 (GDF-15) comme biomarqueur de dysfonction cardiaque (Kempf and Wollert 2009), aussi associé au développement d'une néphropathie chronique (Lajer *et al.* 2010; Ho *et al.* 2013). Nous avons suivi l'évolution de la fonction rénale au long du traitement par la mesure de biomarqueurs et du taux de filtration glomérulaire en fin de traitement, et de la fonction cardiaque par l'utilisation de l'échocardiographie. De plus, l'expression de certains gènes que nous pensions pouvoir être induits par l'AngII a été évaluée au niveau du rein. Dans une seconde cohorte d'animaux, le même schéma expérimental a été reproduit et nous avons testé les effets protecteurs de l'antagoniste des récepteurs aux minéralocorticoïdes éplérénone, et d'un inhibiteur de l'aldostérone synthase, sur les fonctions cardiaque et rénale.

Introduction

This work consisted in 2 studies. We first tempted to improve and characterize a cardio-renal rat model for human diabetic nephropathy based on the ZSF1 model, with translational readouts measurable in a short development time-frame. We aimed at exacerbating and accelerating the existing cardiac and renal defects observed in ZSF1 rats. To do so, we induced both hemodynamic and hormonal stress *via* blood pressure increase and elevation of aldosterone levels with a 6-week subcutaneous infusion of AngII. We expected impairment of systolic function. We used Growth Differentiation Factor-15 (GDF-15) as a biomarker for heart failure, as reported in the literature (Kempf and Wollert 2009). This is a member of the TGF family that is poorly expressed in many tissues in normal conditions. Moreover, high circulating levels of GDF-15 are associated with faster deterioration of kidney function and prediction of incident chronic kidney disease (Lajer *et al.* 2010; Ho *et al.* 2013). It was also shown in T2D patients that GDF-15 could predict elevation of albuminuria (Hellemons *et al.* 2012). We focused on kidney function and structural changes, biomarkers degradation. We checked some selected genes that could be impacted by AngII infusion, to get a hint on the pathways involved in the exacerbation of the cardio-renal disease in rats.

In a second study, we reproduced the previous synopsis, and evaluated the protective effects of an in-house selective ASi in comparison to those of the mineralocorticoid receptor antagonist eplerenone.

Experimental study 4 (*Kidney International*, to be submitted in April)

Aldosterone synthase inhibitor reverses kidney damage in a novel diabetic and cardio-renal rat model

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Key words: Blood pressure; Nephropathy; Cardiomyopathy; Echocardiography; Angiotensin II; Metabolic syndrome; ZSF1 rat model.

Running Title: Heart and renal functions of Angiotensin II infused ZSF1 rats.

Abstract

Cardiac failure and chronic kidney disease are recurrent death causes observed in metabolic syndrome and Type 2 Diabetes patients. Despite huge unmet medical needs, no translational cardio-renal animal model has been established that could support new drug discovery. We designed and evaluated a new rodent model by infusing Angiotensin II (AngII) to ZSF1 rats. This new "ZSF1-AngII" rat model is characterized by late- and complex onset of metabolic syndrome. Renal structure and function of ZSF1-AngII rats was systematically characterized by glomerular filtration rate (GFR) measurement, echocardiography, histopathology examination, measurement of selected plasma biomarkers and gene expression profiling. ZSF1-AngII rats, are characterized by significantly elevated aldosterone levels (64 fold), reduced GFR (by 55%), reduction of ejection fraction (by 36%), microscopic structural changes, worsening of kidney biomarkers and dysregulation of relevant biological networks, that finally mimicked features of human diabetic nephropathy and cardiomyopathy. A proof-of-concept study, showed that ZSF1-AngII rat model combines cardiac and renal defects that were exacerbated compared to ZSF1 rat model, mainly driven by activation of the Renin Angiotensin Aldosterone System. In a second characterization step, we evaluated the efficacy of a novel selective aldosterone synthase inhibitor (ASi), in cardiac and kidney functions of the ZSF1-AngII model. We found that the selected ASi reversed renal damages better than eplerenone, an established aldosterone receptor antagonist. In conclusion, we demonstrated that ZSF1-AngII rat constitutes an accurate translational model to human diabetic cardio-renal syndrome and represents a novel tool to evaluate cardio-renal impacts of existing and innovative new therapeutics.

Introduction

Type 2 diabetes (T2D) is a global epidemic, affecting 311 million people worlwide (WHO report; [1]). There is an unmet need for medication curing or preventing the development of T2D and related co-morbidities, which mainly involve cardiovascular and renal systems [2,8]. This objective is rendered difficult by the lack of appropriate animal models translating complex disease features and drug efficacy. Identification of models combining cardiac and renal symptoms would fill current high medical needs and allow rigorous evaluation of disease-modifying new drugs acting on cardiac and renal dysfunction.

The obese, diabetic ZSF1 rat (Charles River, Wilmington, USA) is a F1 hybrid model developed by crossing rat strains characterized by two different leptin receptor mutations (fa and fa^{cp}). ZSF1 is a rat model of metabolic syndrome that progressively develop diabetic nephropathy (DN) and to a lesser extent heart dysfunction although with preserved ejection fraction (EF) [4,5,6,14,33]. In 2011, Bilan *et al.* showed that ZSF1 rat was considered as the most appropriate rat model of DN, displaying common features of human disease as recommended by the Animal Models of Diabetic Complications Consortium [9,10]. However, despite some similarities with human DN, ZSF1 model misses several important features of renal and cardiac dysfunctions: end-stage renal failure emerges lately (at around 12 months of age) [32], EF is preserved, and diastolic function is altered to a minimal extent along aging [6], as compared to human patients. Obviously, a more severely exacerbated model is needed to capture main features and extent of human DN.

We consider that several key features of human DN that deserved to be captured by a transitional rodent model. Deterioration of biomarkers (BMs) that are translational for kidney function, such as albumin, kidney injury molecule 1 (KIM-1) [12,13,14,15], β2 Microglobulin (β2M), Cystatin C (Cyst C) [16,17,14], TGF-β1 [18,19,20,21], and GDF-15 [22,23,24,25], should be demonstrated altered. Actually, these BMs, derived from the clinical observations and used in population stratification [26] are powerful tools for evaluating disease severity as well as drug efficacy.

Physiopathologically, increased renin angiotensin aldosterone system (RAAS) activity is another essential feature to recapitulate in a translational model of DN since its dysregulation plays an important role in the pathogenesis of cardiovascular and renal disorders [27, 28]. In particular, AngII-induced hypertension leads to impaired renal function and renal injury [29]. Moreover, drugs that block AngII are renoprotective primarily through antiproteinuric action at the level of podocytes [30]. Unfortunately, existing data in ZSF1 model missed to demonstrate deterioration in RAAS activity, making ZSF1 rats missing one pivotal feature of human DN [9].

In this study, we tempt to develop and characterize a novel cardio-renal rat model for human DN with translational readouts measurable in a short development time-frame. We aimed at exacerbating and accelerating the existing renal defects observed in ZSF1 rats. To do so, we infused ZSF1 rats with AngII, in order to induce both hemodynamic and hormonal stress due to blood pressure (BP) increase and elevation of aldosterone levels. Under these experimental conditions, we expected impairment of EF and exacerbation of kidney damages in a faster and a greater extent than without AngII infusion.

We performed selected experimental interventions in the new model (ZSF1-AngII) to evaluate its capacity of capturing human T2D pathology and associated co-morbidities. We observed similar cardiac and renal dysfunction as observed in DN patients. ZSF1-AngII rat can be as a model of translational DN and be useful for new drug assessment and development. Finally, with this model, we showed that a novel potent selective inhibitor of aldosterone synthase is more efficacious in reverting kidney and heart damages in ZSF-AngII rats than the reference compound eplerenone.

Material and Methods

Animals and protocols

Male ZSF1 [(OB) Gmi Crl-fa/fa] were ordered at the age of 12W from Charles River U.S. and directly fed a special enriched diet (PURINA PMI 5008, Ssniff R/M-H, Germany, 10 mm). Rats were housed 4 per cage (Tecniplast®, Italy, Eurostandard Type IV, model 1354G, size: 595mm x 380mm x 200mm) at approximately 21°C and 55–65% relative humidity, and had *ad libitum* access to food and water. Light cycle was from 6 a.m. to 6 p.m.

Experimental procedures were performed under animal research protocols (ARP) validated by F. Hoffmann-La Roche, the Swiss Cantonal Veterinary Office Basel-Stadt and in accordance with guidelines of the Association for the Assessment and Accreditation of Laboratory and Animal Care (AAALAC # Unit Number: 001057).

Two cohorts of 26 week-old male ZSF1 rats were used:

- One cohort was used to characterize the ZSF1-AngII rats as a cardio-renal model. In this cohort rats were treated with either Vehicle, AngII (100ng/kg/min) or Lisinopril (30 mg/kg/day).
- The second cohort of ZSF1-AngII rats was constituted of four groups submitted to Vehicle, AngII (100ng/kg/min), AngII (100ng/kg/min) + ASi (20mg/kg/day), AngII (100ng/kg/min) + eplerenone (200mg/kg/day).
- Treatments were given as food admix (compounds mixed with the PURINA PMI 5008 food). AngII was infused continuously via osmotic pumps as described below for 6 weeks.

Rats were stratified in homogenous groups based on albuminuria, urine cystatin C (uCyst-C) levels, and body weight (BW).

Blood pressure monitoring

Systemic arterial BP measurement was performed non-invasively in conscious animals by using tail-cuff method (based on the Volume Pressure Recording technology, Kent Scientific Corporation, USA, Model CODA) [31]. Systolic (SAP) and diastolic (DAP), arterial pressure were measured 2 hours after the animals have been acclimated to a dedicated room heated at 28°C. Mean arterial pressure (MAP) was calculated from SAP and DAP measurements. The first SAP and DAP measurement performed in ZSF1 rats took place the day before the surgery, then every 2 weeks, e.g. at 28W, 30W, and 32W of age.

Surgical procedure

Rats received analgesia 1h prior to surgery (Buprenorphine 0.05 mg/kg s.c.), then were anesthetized with isoflurane (Forène®, Abbott Laboratories, Queenborough, UK). Rats' body temperature was maintained at 37°C.

A sub-cutaneous pocket was created by blunt dissection and an osmotic pump was inserted (ALZET®, USA, type 2006, 0.15µl/h, reservoir volume 200µl), filled with AngII solution (Sigma–Aldrich®, USA). The skin incision was closed with a-traumatic wound staples. After the surgery, the animals were single housed for at least one week to let them recover and received analgesia treatment (Buprenorphin 0.05 mg/kg. s.c.) twice a day for two days. After recovery, the wound staples were removed and the animals returned to group housing.

Urine, blood sampling methods and Biomarkers measurements

Urine was first collected one week prior to surgery for stratification on selected markers, then every 3 weeks, e.g. at 29W, and 32W of age. Rats were placed in metabolic cages. Urine was collected for

24 hours, then sampled and centrifuged (800RPM for 10 min at 4°C). Body weight (BW) was recorded regularly along the aging and treatment period.

Blood was collected from anaesthetized rats (isoflurane) at the end of the study by decapitation (500µl) and dispatched into tubes coated with EDTA (Sarstedt AG & Co., Germany, Microvette®500), containing 1% aprotinin (to inhibit protein degradation) and kept on ice. Tubes were then centrifuged (10 000 RPM for 15 min at 4°C) and plasma was aliquoted in different tubes or 96-well plates for analysis. Blood samples were taken under non fasting conditions in the morning 2h following switch from dark to light cycle. BMs that represent kidney Integrity or function were measured in urine: TGF-ß1 (ELISA, BioLegend, USA, Legend-max 436708); β2M (ELISA, Mitsubishi, Japan, PRH111); Cystatin C (ELISA, R&D Systems, USA, MSCTC0); GDF-15 (ELISA, Cusabio, China, CSB-E16317r); KIM-1(ELISA/LUMINEX, Millipore, USA, RKTX1MAG-37K); Albumin (ELISA, Alpco, USA, 41-ALBRT-E01); other BMs for kidney function were measured in plasma: Renin (RIA, DiaSorin, Italia, P2721); Angiotensin 2 (RIA, DRG international, USA, RIA-3020); Creatinine (COBAS, F.Hoffmann La-Roche AG, Roche Diag./ 03263991 190); fluorescein isothiocyanate (FITC)-Inulin (adapted from the method described by Qi and Breyer, 2009 [32]); Aldosterone (LC-MS, Waters, USA, Xevo TQ MS).

Measurement of glomerular filtration rate

Measurement of glomerular filtration rate (GFR) was performed during the 6th week of treatment, when rats were 32W-old. FITC-inulin (Sigma–Aldrich®, USA, 40 mg/mL in 0.9% NaCl solution) was administered i.v. at a volume of 1 mL/kg in one tail vein.

At 0.5, 3, 7, 10, 15, 20, 40 and 60 min after administration, blood (~60µL) was collected by tail nick in EDTA-coated tubes (Sarstedt AG & Co., Germany, Microvette®300) containing 1% aprotinin and kept on ice until centrifuged then analyzed.

Echographic assessment of cardiac structure and function

Cardiac function was measured non-invasively in anesthetized rats (Isoflurane/O2 (induction 5% Isoflurane, then 2%) by echocardiography using a Vevo 2100® system (Visualsonics, Canada) coupled to a MS 201 linear scan head; the air was humidified to avoid dryness of the respiratory tract during the procedure. The whole intervention did not exceed 20 minutes. Isoflurane was chosen among the existing anesthetics because it was shown to alter the least cardiac function [33]. Nevertheless, we are aware that isoflurane directly depresses myofilament contractility by decreasing Ca^{2+} responsiveness [34]. Heart rate and body temperature were monitored in real time on the Vevo 2100®, which enabled us to monitor the stability of anesthesia throughout the intervention, and maintained constant body temperature at 37°C.

Echocardiography examination was performed as follows: In M-mode and parasternal short axis view, the principal left ventricular dimensions were recorded, *i.e.* left ventricular internal diameter in diastole and systole (LVIDd, LVIDs), left ventricular anterior wall in diastole and systole (LVAWd, LVAWs), interventricular septum in diastole and systole (IVSd, IVSs). Each echocardiographic exam was performed in triplicate and measurements were done on 3 different pictures using the reporting program included in the machine. An average of the 3 measurements was done to increase the reliability of the data. Left ventricular mass (LV mass) was calculated according to the following corrected formula adapted to rodents:
$1.05x((LVID_d+LVPW_d+IVS_d)^3 - LVID_d^3) \times 0.8$ [35]. Ejection fraction (EF) was calculated to assess the systolic function.

Histology

At necropsy, kidney and heart were harvested and immediately immersed in 10% neutral buffered formalin for maximum 24h. After being rinsed, the tissues were embedded in paraffin blocks and

slices of 4µm were prepared that were stained with standard hematoxylin eosin stain (HE; all tissues) and Periodic acid-Schiff reaction (PAS; kidney only).

Scoring system for kidney structures changes (glomerular vacuolation, thickening of basement membranes, interstitial inflammation, interstitial fibrosis, tubular dilation, proteinaceous casts) was established as follows: grade 0 (no change), grade 1 (minimal; ≤ 10% of a structure affected), grade 2 (slight; between 10 and 20% of a structure affected), grade 3 (moderate; between 20 and 50% of a structure affected), grade 4 (marked; between 50 and 75% of a structure affected), and grade 5 (severe; up to 100% of a structure affected).

For glomerulosclerosis (GS), 50 glomeruli per kidney were examined and each scored into grade 0 (no GS), grade 1 (≤ 25% of glomerulus affected), grade 2 (≤ 50% of glomerulus affected), grade 3 (≤ 75% of glomerulus affected), and grade 4 (up to 100% of glomerulus affected). For the calculated GS score for each kidney, the sum of each glomerulus multiplied with the score was calculated, and the obtained sum was graded as follows: grade 1 (sum ≤ 25), grade 2 (sum between 26 and 50), grade 3 (sum between 51 and 75) grade 4 (sum between 76 and 100) grade 5 (sum > 100).

Gene analysis procedure

After the sacrifice, kidneys and heart were quickly harvested and sliced longitudinally in RNAlater® (Ambion, USA). Medulla was removed and large pieces of cortex were cut, placed in tubes (Eppendorf. Germany, 2ml tubes) with 5-10 volumes of RNAlater® stored at 4°C than -20°C until use. Tissue was homogenized using Tissue Lyser (Qiagen, Germany) methodology: One steel bead and 20mg of cortex stored in RNAlater® solution (Ambion, CA, USA) were added to 1ml of RLT-plus Lysis buffer, put on ice and homogenized for 2x 2 Min at 20Hz, followed by 2 Min centrifugation. 400µl supernatant were subjected to automatized RNA extraction protocol using the QIAsymphony (19585, S-Blocks 96) with final elution volume of 100µl.

50ng of total RNA were subjected to the NuGen Ribo-SPIA TM amp (NuGen, CA, USA) protocol in first and second strand cDNA synthesis coupled with SPIA Amplification protocol according to

manufacturer's instructions. 3µl of Ovation TM WB reagent were added to 79µl of SPIA reaction and incubated at 48°C for 30 Min. Purification of amplified cDNA was performed using the QiaQuick PCR Purification Kit (Qiagen, Germany) according to manufacturer's instructions in elution volume of 30µl. In brief fragmentation and biotin labelling were performed using the Encore Biotin kit (cat. 4200; NuGen, USA) with 4.4µg cDNA in 25µl buffer, mixed with 7µl of the Fragmentation Master Mix followed by addition of 18µl of the Labelling Mix according to manufacturer's instructions. Selected samples were quality checked for grade of cDNA fragmentation on Agilent Bioanalyzer.

For Hybridization and Cocktail assembly, the HWS kit (Affymetrix, UK) was used with 50µl of fragmented, biotinylated cDNA according to manufacturer's instructions. Hybridization, Wash and Stain were performed using the respective Affymetrix kit (cat. 900720), Rat Genome 230 2.0 arrays and a Fluidics-450 station.

RNA extracts were profiled with Affymetrix RAT 230 2 chip (Santa Clara, CA). Quality control (QC) was performed with an internal software pipeline and chips with quality issues were removed from subsequent analysis. Expression signals were normalized by the RMA method as implemented in the *affy* package in R/Bioconductor.

Differentially expressed genes were identified by linear models and the empirical Bayes method implemented in *limma* [36].

Pathway analysis was performed with the gene set enrichment (GSE) algorithm *camera* [37]. Gene sets in use were derived from an internal database integrating internal and public resources including the Reactome database and the NCI pathway interaction database. For both gene-level and pathway-level analysis, multiple-testing adjustment was performed with the Benjamini-Hochberg method to control false-discovery rates [38].

Statistical analysis

Fisher test was used to test homoscedasticity. Some ZSF1 parameters were only assessed at the end of the study (e.g. at age 32W) and others were longitudinally monitored between 26W and 32W.

For parameters measured at 32 W only (bar graphs), a 1-way ANOVA followed by Dunnett's post-hoc test was used; * p <0.05; ** p <0.01; *** p <0.001 vs vehicle group, and **iii** p <0.001 vs. AngII group.For parameters measured longitudinally (curve graphs), MANOVA with multiple comparisons was used; # p <0.05; ## p <0.01; ###p <0.001 vs vehicle group, and $ p <0.05; $$ p <0.01; $$$p <0.001 vs AngII group.

Analysis were all performed using JMP8 software (SAS institute Inc., USA), otherwise stated differently.

Results

AngII-infused ZSF1 rat model

MAP measurement and impact of treatment on RAAS of AngII-infused and lisinopril-treated ZSF1 rats

MAP measured in vehicle group was relatively stable from age 26W-to32W, and moderately elevated (144 mmHg and 126 mmHg, respectively). In AngII-treated group and two weeks after the infusion started (e.g. from age 28W), MAP was significantly higher compared to vehicle-treated rats and reached 183 mmHg at 32W vs. 126 mmHg that correspond to 45% increase in MAP compared to age-matched rats in vehicle group. In contrast, lisinopril induced 7% decrease in MAP compared to vehicle after 6 weeks of treatment reaching 117 mmHg at 32W of age, (Figure 1A) while maximal efficacy was observed after 4 weeks of treatment (-20%).

Renin activity evaluated in plasma was decreased by -72% in AngII-treated rats, and was significantly increased by +2730% by Lisinopril compared to vehicle as measured at 32W of age (AngII:0.37±0.10; Lisinopril: 35.76±3.04 vs. Veh:1.31±0.18 ng/ml/h) (Figure 1B). AngII plasma levels were strongly increased by + 1164% in the AngII-treated group and by +364% in the lisinopril group compared to vehicle (AngII: 74.9±18.3; Lisinopril: 16.4±1.7 vs. Veh: 4.5±1.4 ng/ml) (Figure 1B).

Finally, Aldosterone plasma levels were significantly increased by +6472% by AngII and decreased by -90% by lisinopril as compared to vehicle (AngII:7.12±2.04; Lisinopril: 0.01±0.00 vs. Veh: 0.11±0.03 nM) (Figure 1B).

Changes in RAAS hormones in 32W-old ZSF1 rats observed after 6 weeks of treatment paralleled changes in adrenal structure. IHC analysis of adrenals showed vacuolation and hypertrophy of adrenal cortex (mainly zona glomerulosa) of rats treated with AngII (Figure 1C). This structural modification is considered a consequence of the stimulation of aldosterone synthesis and its trophic role that stimulates zona glomerulosa [27].

Renal structure and function of AngII-infused and lisinopril-treated ZSF1 rats

GFR was significantly reduced (-55%) after 6 weeks of AngII infusion compared to vehicle, while lisinopril showed a trend in improving it (+16%) (for AngII: 0.44±0.07; for Lisinopril: 1.14±0.07 vs. Veh: 0.98±0.09 ml/min/g kidney) (Figure 2A).

Figure 2B shows that albuminuria constantly increased from 403 mg/24h (at 26W of age) to 791 mg/24h (at 32W of age) in the vehicle group suggesting kidney function deterioration along aging in ZSF1 rats. Albuminuria was further increased by AngII infusion from 400 mg/24h to 1183 mg/24h from 26W- to 32W of age, corresponding to +50% increase compared to Vehicle. Lisinopril highly significantly reduced albuminuria from 410 mg/24h to 173 mg/24h corresponding to -78% compared to vehicle. MANOVA analysis showed a significant difference in albuminuria evolution between vehicle and AngII and vehicle vs. Lisinopril ($p(time*treatment)<0.001$). AngII deteriorated albuminuria along aging of ZSF1 rats while lisinopril prevented its worsening. Another way to estimate kidney function is the measurement of creatinine clearance. In our study, creatinine clearance was significantly decreased by -73% by AngII in 32W old ZSF1 rats, while creatinine clearance was slightly decreased by Lisinopril, by -14%, as compared to vehicle.

Changes in albuminuria, GFR and creatinine clearance (Figure 2A-B) indicate that AngII significantly deteriorated kidney function while lisinopril prevented deterioration induced by aging. Similarly, urine BMs that translate kidney function were significant increased by 2-4 fold (deterioration) by AngII while lisinopril induced a significant 2-fold decrease (improvement) of the measured parameters (Table1).

In addition to changes in kidney function and translational BMs, AngII infusion induced GS, interstitial fibrosis, tubular dilation and proteinaceous casts more than observed in vehicle-treated animals along aging. On the opposite, lisinopril reduced GS, thickening of basement membrane, interstitial inflammation and fibrosis, tubular dilation, and proteinaceous casts of ZSF1 rats. No difference in glomerular vacuolation was observed between vehicle, AngII and lisinopril (Figure 2C).

Cardiac function of AngII-infused and lisinopril-treated ZSF1 rats

Cardiac function of ZSF1 rats was assessed by echocardiography. As displayed in Figure 2D, contractility as depicted by measurement of ejection fraction (EF) was significantly decreased after 6 weeks of AngII infusion by (-36% versus Vehicle), while, EF was unchanged during treatment with Lisinopril (AngII: 47±4, $p<0.001$; Lisinopril: 71±2 vs. Veh: 73±1 %, NS).

LV mass/BW ratio was significantly increased by +34% by AngII compared to Vehicle, whereas Lisinopril did not impact this ratio (AngII: 2.66±0.16; Lisinopril: 2.09±0.07 vs. Veh: 1.98±0.05).

Gene expression profile of AngII-infused and lisinopril-treated ZSF1 rats

From functional and histology data reported in Figures 1-2, it was strongly suggested that AngII and lisinopril act the opposite way onto renal and cardiac function of ZSF1 rats. Kidney gene expression profile (GEP) was performed in order to depict different mode of action of both drugs. Both AngII and Lisinopril induced major differential gene expression pattern compared to vehicle as shown in plots of Figure 3A. Microarray analysis (Figure 3 B-E) suggested that lisinopril exert an opposed effects to that induced by AngII, in line with the mode of action of ACE inhibitor. Moreover, pathway enrichment analysis showed that AngII induced overexpression in metabolic cluster, notch cluster, TGF-beta/SMAD pathway, glucocorticoid pathway, and that lisinopril induced expression changes in cell cycle cluster, metabolism cluster, MAPK pathway and c-Myc pathway. Overall, lisinopril effects are opposite to AngII's overexpressing effects on the metabolic cluster and the notch cluster and also negatively regulated GPCR and PLC-gamma pathway compared to AngII. These data strongly suggest that AngII and Lisinopril exert opposite effects in terms of global gene expression.

Pathway analysis (Figure 3B-D) indicates that notch signalling may participate in the biological process as already described in 2011 by Sharma et al. [39]. Other involved pathways include TGF-β, cell metabolism, cell cycle and glucocorticoid pathway, indicating potential involvement of immune pathways since the glucocorticoid pathway is a feedback mechanism of immune activities (Figure 3).

Test of compounds related to aldosterone (eplerenone - ASi) in AngII-infused ZSF1 rats

Kidney and cardiac functions

Cardio renal ZSF1-AngII rat model was further explored in a second study wherein set of treatment including vehicle (without AngII infusion), AngII, AngII + ASi and AngII + eplerenone. Efficacy of treatments on cardiac and renal functions was evaluated by several types of interventions.

Infusion of AngII significantly increased plasma aldosterone levels in ZSF1 rats compared to vehicle (by 33 fold, $p<0.01$) (Figure 4A). Treatment with ASi prevented AngII-induced increase in aldosterone by -99%. Treatment with eplerenone was not efficacious in preventing AngII effects on aldosterone levels. (AngII: 13.64±0.79; AngII + ASi: 0.16±0.03; AngII + eplerenone: 15.18±2.46 vs Veh: 0.04±0.01 nM).

In study 2, MAP measured in vehicle and AngII group (for Veh: 149 mmHg, for AngII: 194 mmHg) were very similar to that measured in study 1 (for Veh: 144 mm Hg, for AngII: 183 mm Hg) (cf. Figure 1). In AngII, AngII + ASi, AngII + eplerenone groups, MAP was increased to a similar extent (by around 35%) compared to vehicle from 28W of age and was maintained around 185 mmHg till 32W of age (data not shown). No difference in MAP in-between the 3 treatment groups was observed (data not shown).

GFR was significantly reduced (by -58%) after 6 weeks of AngII infusion as compared to vehicle ($p<0.001$) (Figure 4B). Eplerenone given on top of AngII infusion induced a decrease in GFR of -26%, whereas ASi given on top of AngII infusion induced a full protection against loss of glomerular function (AngII: 0.69±0.10; AngII + ASI: 1.62±0.0; AngII + eplerenone: 1.21±0.11 vs Veh: 1.63±0.19 ml/min/g kidney). As a result, AngII + ASi group was statistically different compared to AngII group ($p<0.001$) (Figure 4B).

In this second study, albuminuria levels stayed relatively stable in the vehicle group (from 398 mg/24h at 26 W to 338 mg/24h at 32W). When compared with study 1, albuminuria measured in 32W-old ZSF1 rats of study 2 is much lower (338 mg/24h, in study 1 vs. 791 mg/24h in study 2). This difference would suggest that kidney function measured before AngII infusion in the second ZSF1 rat cohort was less severely degraded as compared to ZSF1 rat cohort in study1. Infusion of AngII induced a significant raise of albuminuria from 463 mg/24h (at baseline) to 568 mg/24h (at 32W) with a peak reached at 29W of age (810 mg/24h) corresponding to 254% increase compared to vehicle (Figure 4C). Treatment with ASi on top of AngII nearly blunted the increase in albuminuria induced by AngII keeping its level from 394 to 483 mg/24h.

At 32W, AngII-induced increase in albuminuria was +68% compared to vehicle. Treatment with eplerenone induced only a partial protection against albuminuria increase induced by AngII after 3 weeks of treatment. Then eplerenone protective effect on albuminuria was lost after 6 weeks of treatment (at 32W of age) (Figure 4C). Treatment with ASi reduced albuminuria increase to +43%, while eplerenone induced similar increase as AngII alone (+77%) suggesting absence of sustained efficacy in preventing albuminuria increase by AngII infusion.

Overall, MANOVA showed a significant difference in albuminuria evolution between vehicle and AngII ($p(time*treatment)<0.001$); a significant difference between vehicle and AngII + eplerenone ($p(time*treatment)<0.01$); no difference between vehicle and AngII + ASi ($p(time*treatment) = 0.12$), and finally a significant difference between AngII and AngII + ASi ($p(time*treatment)<0.001$).

Creatinine clearance was significantly decreased by -84% by AngII at 32W, when compared to vehicle, confirming the role of AngII in worsening kidney function. Treatments with ASi or eplerenone were poorly efficacious in preventing decline in creatinine clearance (-71% and -75% respectively) (Figure 4).

Changes in kidney function induced by AngII and effect of treatments (ASi and eplerenone) described in Figure 4 (A-D) also impacted urine BMs that mainly represent kidney function. Changes in kidney BMs measured at the end of the chronic treatment of study 2 are summarized in Table 2. Overall, AngII and AngII + eplerenone showed a consistent and significant 2.5-3 fold increase of the selected BMs. Treatment with ASi showed only 1.5-2 fold increase of the measured parameters compared to vehicle. Efficacy of ASi in preventing BMs deterioration induced by AngII was significant when compared to AngII alone.

In parallel to deterioration of kidney function (GFR decline) and selected BMs, AngII infusion showed tendency to worsen GS and tubular dilation compared to vehicle animals as assessed semi-quantitatively at the end of the chronic treatment (Figure 4D). Treatment with ASi or eplerenone showed a tendency to similarly protect kidney for score deterioration as shown by kidney score close to that of vehicle group (non-infused with AngII) (Figure 4D). It has to be noticed that most of the kidney structural changes observed after AngII are relatively modest after 6 weeks of treatment that limits evaluation of treatment's efficacy on kidney structure.

Impact of AngII and treatments (eplerenone and ASI) was evaluated not only on kidney but also on heart. Systolic function is measured by EF. As shown in Figure 4E, EF was significantly decreased by AngII (-28%) compared to vehicle ($p<0.01$). Treatment with eplerenone did not prevent EF decline (-26% compared to vehicle, $p<0.01$). Treatment with ASi showed a trend in reducing decline in EF induced by AngII (AngII: 52±4; AngII + ASi: 60±4).

LV mass/BW ratio was increased by both AngII and AngII + ASi treatments as compared to vehicle (not significantly) (Figure 4E). In contrast, AngII + eplerenone slightly reduced it (AngII: 2.65±0.17; AngII + ASi: 2.53±0.22; AngII + eplerenone: 2.02±0.05 vs Veh: 2.26±0.14) (Figure 4). These data suggest a different impact of ASi and eplerenone on cardiac mass and cardiac function.

Modification of Gene expression

Pathway enrichment analysis (Figure 5A) showed that AngII induced a significant overexpression of genes belonging to cell cycle, notch, TGF-β and glucocorticoid pathways. These data are similar to those obtained in the first study (see figure 3A). We observed striking differences in how GEP evolved in the three organs (kidney, heart and adrenals) when treated with eplerenone and ASi on top of AngII. In each organ examined, AngII dramatically changed GEP as shown by the distinct circles from that of vehicle group (Figure 5A). In kidney, eplerenone only tended to decrease the overexpression of the main four pathways overexpressed by AngII, whereas ASi significantly dropped these expression patterns (Figure 5B). We observed an exception for the glucocorticoid pathways in which a huge reduction of gene expression occurred under AngII infusion. None of the treatments impacted these pathways in the heart (Figure 3A).

Discussion

As reported in literature, male ZSF1 rats start developing metabolic syndrome with moderate high blood pressure at relatively early development stage (*i.e.* around 12W of age), then develop renal failure lately, around 52W of age [32]. In a precedent work, we showed that cardiac function in ZSF1 rats started to worsen around 18W of age, although only diastolic function was significantly impacted [6].

In this work, we show that chronic subcutaneous AngII infusion in 26 weeks-old male ZSF1 rats (for six weeks) led to significant increase in circulating aldosterone and significantly impaired cardiac and renal functions, to an extent that recapitulates DN and associated co-morbidities in humans. Our work demonstrates that AngII infusion exacerbated existing pathologies described in ZSF1 rat model [33,32]. Moreover, treatment with AngII for 6 weeks strongly reduced the time frame needed to observe renal impairment since significantly more severe kidney dysfunction (decline in GFR, Crea

clearance and BMs) was achieved at 32 weeks of age instead of one year of age. Then, systolic impairment was induced by AngII infusion, while EF was kept unchanged in ZSF1 rats until one year of age.

The significant reduction of time frame to achieve worsening of kidney function in the ZSF1 rat model and the induction of cardiac dysfunction can represent a pivotal model for supporting optimal drug discovery. The impact of AngII in increasing circulating levels of aldosterone (and other steroids) is another feature that is ultimately necessary to assess the efficacy of RAAS-related compounds and also optimally characterize selectivity of ASi. Aldosterone is recognized for its role in regulating potassium and sodium levels via mineralocorticoid receptors (MR), found in the collecting ducts of the kidney and also in other tissues (adipose tissue, vascular smooth muscle cells or vascular endothelium). Aldosterone also induces reactive oxygen species, inflammation and fibrosis, via MR inducing among others nuclear factor κB (NF-κB) or Serum and Glucocorticoid-regulated Kinase 1 (SGK1), both belonging to the Glucocorticoid pathway [40]. These effects negatively impact kidney function, by inducing pro-oxidative stress, triggering genotoxic effects and inflammatory pathways leading to fibrosis [41, 42]. As reported by Ichikawa *et al.* in 2014, the first line of treatment for patients with CKD (like DN), is represented by Angiotensin Converting Enzyme inhibitors (ACEi) and AngII receptor blockers (ARBs). None of the existing ACEi or ARBs can suppress either the progression of the disease or aldosterone secretion in the long run [43,44]. Other strategies to tackle DN progression are needed. To date, MRa like spironolactone or the more selective drug, eplerenone, can prevent interstitial fibrosis and/or CKD in kidney injury rat models [45,46]. However, MRa is enable to prevent the non-genomic effects of aldosterone (e.g. not *via* MR) mediated via effects on protein kinase C, intracellular calcium and cyclic adenosine monophosphate [47]. As a result, it was suggested that a selective ASi could better protect targeted organs during DN than MRa [42].

AngII was chosen to exacerbate kidney dysfunction via its effect on systemic pressure and its impact on RAAS which was found modulated in chronic kidney disease [48]. AngII dramatically increased MAP in both studies conducted by about +40%. As a result of its mode of action, the RAAS was enhanced as demonstrated by increase in aldosterone levels and other corticoids, as well as increase in renin activity (Figure 1B). Adrenal cortex (mainly zona glomerulosa) was strongly remodeled which is the consequence of the overproduction of aldosterone that acts as a trophic hormone. Both hemodynamic and hormonal stress triggered by AngII led to renal and cardiac structure remodeling and dysfunctions (Figure 1). Deterioration in renal structure (worsening of kidney score) paralleled deterioration of kidney BMs, creatinine clearance and decrease in GFR, indicating deterioration of kidney function. For the first time in a complex rodent model of metabolic defects (ZSF1), we showed that that significant decline in cardiac function coexisted with dramatic worsening of kidney function. Actually, a drop of more than 35% in systolic function was measured in male ZSF1 rats after only 6 weeks of AngII infusion. Diastolic function (mainly assessed by the measurement of E/A ratio) was not measureable in AngII-treated animals since E wave was increased but no A was measurable. As it is reported that systolic function is altered after the diastolic function we could assume that ZSF-AngII rats would be characterized by strongly deteriorated diastolic function (if it would have been measurable) [5,29]. Conversely to hypertensive effect of AngII, standard of care, lisinopril, reduced blood pressure of ZSF1 rats and tended to protect kidney structure and function, without changing cardiac function.

In order to characterize the ZSF1-AngII cardio-renal model, we performed a second study in ZSF-AngII rats treated with either MRa eplerenone or an in-house CYP11B2 selective ASi (assessed in vitro and in vivo, in rats and monkeys). The aim was to compare the efficacy of our in-house selective ASi with the standard of care eplerenone. In this cardio-renal model, ASi induced strong protection of renal structure and function and restored significantly GFR compared to AngII infused rats. Eplerenone was efficacious on renal protection to a less extent to ASi. Both drugs improved kidney BMs. Deterioration of cardiac function induced by AngII was minimally prevented by ASi while

eplerenone did not show cardio protection. The lack of significant cardio protection by both treatments could be due to the limited treatment duration (6 weeks) or could suggest that additional mode of action would be needed. Nevertheless we demonstrated that MRa such as eplerenone can partially counteract the negative effect of AngII at least on kidney function. The selective ASi demonstrated a superior efficacy compared to eplerenone on kidney function. Gene expression profiles confirmed the specificity and superiority of ASi treatment over eplerenone.

If we have a closer look to the renal BMs values measured in the two studies, we captured a difference in parameters measured in controls and in AngII infused animals especially regarding albuminuria. Rats were ordered from the same provider, were fed and stored under comparable conditions and treatment started at supposing the same age (26W). According to provider information, rat age is guaranteed plus or minus 1 week, suggesting that we could have at maximum up to two weeks of age difference between the two slots. According our measurements, we estimated that ZSF1 rats used in study 2 -in which ASi and eplerenone were tested-, were younger than ZSF1 rats used in the study 1. This difference would explain that the less dramatic deterioration in albuminuria along aging in the second study, and that overall the BMs values are inferior in this cohort of rats compared to those measured in study 1. This hypothesis is reinforced by the fact that GFR value measured in vehicle group is higher in the second study (less kidney function deterioration) than in the first one. In study 2 and under AngII infusion, deterioration of cardiac and renal functions was minimal between 12W to 18W of age (data not shown), despite a comparable elevated blood pressure. Taken together, these data suggest that exacerbation of metabolic syndrome, cardio-renal syndrome by AngII infusion require already established functions deteriorations. In spite of absence of absolute reproducibility in kidney function deterioration between the two slots of rats, we clearly showed that ZSF-AngII rat model is suitable to assess protective effect of ASi.

Kidney protection induced by ASi was superior compared to eplerenone, while MAP values were similarly elevated in both studies. As a result, hemodynamic stress in the context of metabolic syndrome cannot explain alone damages caused in kidney, and in heart to a lesser extent.

In our study, we showed that aldosterone plays a central role in kidney dysfunction that was prevented by ASi which blunted its production (Figure 4). In contrast, eplerenone which did not prevent aldosterone increase under AngII infusion, was unable to significantly protect kidney deterioration.

Finally, our data show that in the ZSF1-AngII cardio-renal model, AngII leads to faster and greater renal damages through activation of TGF-β, Notch, and Glucocorticoid and Cell cycle pathways. In kidney, gene expressions of the pathways were significantly induced by AngII, which confirms our previous findings, as well as by AngII + eplerenone. In contrast, ASi successfully reversed the effect of AngII: gene expression levels are comparable to control for three pathways (TGF-β, Notch and Cell cycle), and the induction is lowered in the Glucocorticoid pathway.

In contrast, gene expression of these pathways in the heart is not significantly altered by either AngII or addition of either eplerenone or ASi, confirming distinct mechanism of actions of AngII in the two organs.

Cyp11B2 (aldosterone synthase) is highly expressed in adrenal gland. Its expression in kidney and heart is almost under the detection limit of microarray (data not shown).This could explain why ASi induces strong expression changes in adrenal gland. Moreover, it tends to show that the observed effect of ASi on kidney function is mediated via adrenal gland, meaning by the absence of aldosterone production. Difference in aldosterone synthase expression in heart and kidney could explain why adrenal gland is the only organ where ASi treatment leads to a very different expression pattern as compared to other groups, in which aldosterone is produced (Figure 5). To better characterize the mode of action of ASi, a more detailed /accurate identification of genes involved in this huge expression shift seen with ASi in the adrenal gland could be deserved. A better

understanding of genes' expression modulated by ASi, could support differentiation of new compounds in non-human primates model and favor a better predictability of efficacy in Human beings.

In conclusion, the structural and functional changes observed in kidney and heart of male ZSF1 rat model infused with AngII show that ZSF-AngII constitutes an accurate translational model to cardiac and renal chronic diseases observed in patients with a metabolic syndrome (DN and associated co-morbidities, kidney failure, diabetic cardiomyopathy). This new rodent model is suitable to evaluate cardio-renal protective impact of drugs in a reasonable time frame. With this model, a complete characterization of new drug could be achieved before entering into Primates testing. We demonstrated that the slow development of cardiac and renal disorders of the ZSF1 rat model can be further exacerbated (faster and greater extent of the damages) by increasing blood pressure and elevating aldosterone levels, *i.e.* by boosting the RAAS.

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Conflict of interest

None declared.

References

1. Danaei G, Finucane MM, Lu Y *et al.* National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2·7 million participants. *Lancet* 2011; **378**: 31-40.

2. Grundy SM, Brewer HB Jr, Cleeman JI *et al.* Definition of Metabolic Syndrome: Report of the National Heart, Lung, and Blood. *Circulation* 2004; **109**:433-438.

3. Grundy SM (2008) Metabolic Syndrome Pandemic. Arterioscl Throm Vas **28**: 629-636.

4. Prabhakar S, Starnes J, Shi S *et al.* Diabetic nephropathy is associated with oxidative stress and decreased renal nitric oxide production. *J Am Soc Nephrol* 2007; **18**: 2945-52.

5. Lourenço A, Falcão-Pires I, Cerqueira R *et al.* Abstract 17471: The Obese Zsf1 Rat as a New Model of Heart Failure with Preserved Ejection Fraction Accompanying the Metabolic Syndrome. *Circ Res* 2012; **126**: A17471.

6. Riboulet W, Hainaut E, Verry P *et al.* Longitudinal assessment of heart function of ZSF1 rats by echocardiography. *JEAAS* 2013; **1**: 125-139.

7. Joshi D, Gupta R, Dubey A *et al.* TRC4186, a novel AGE breaker, improves diabetic cardiomyopathy and nephropathy in Ob-ZSF1 model of Type 2 Diabetes. *J Cardiovasc Pharm* 2009; **54**: 72-81.

8. Tofovic SP, Jackson EK. Rat Models of the Metabolic Syndrome. *Meth Mol Med* 2003; **86**: 29-46.

9. Bilan VP, Salah EM, Bastacky S *et al.* Diabetic nephropathy and long-term treatment effects of rosiglitazone and enalapril in obese ZSF1 rats. *J Endocrinol* 2011; **210**: 293-308.

10. Fioretto P, Caramori ML, Mauer M. The kidney in diabetes: dynamic pathways of injury and repair. *Diabetologia* 2008; **51**:1347-55.

11. Tofovic SP, Kusaka H, Kost CK *et al.* Renal function and structure in diabetic, hypertensive, obese ZDFxSHHF-hybrid rats. *Ren Fail* 2000; **22**: 387-406.

12. Han WK., Bailly V, Abichandani R *et al.* Kidney Injury Molecule-1 (KIM-1): a novel biomarker for human renal proximal tubule injury. *Kidney International* 2002; **62**: 237-44.

13. Lock EA. Sensitive and early markers of renal injury: where are we and what is the way forward? *Toxicol Sci* 2010; **116**: 1-4.

14. Fuchs TC, Hewitt P. Biomarkers for drug-induced renal damage and nephrotoxicity-an overview for applied toxicology. *The AAPS Journal* 2011; **13**: 615-31.

15. Kirk R. Renal fibrosis: KIM-1 expression links kidney injury with CKD in mice. *Nat Rev Nephrol* 2013; **9**: 627.

16. Herrero-Morin JD, Malaga S, Fernández N *et al.* Cystatin C and beta2-microglobulin: markers of glomerular filtration in critically ill children. *Crit Care* 2007; **11**: R59.

17. Dieterle F, Perentes E, Cordier A *et al.* Urinary clusterin, cystatin C, beta2-microglobulin and total protein as markers to detect drug-induced kidney injury. *Nat Biotechnol* 2010; **28**: 463-9.

18. Fan JM, Ng YY, Hill PA *et al.* Transforming growth factor-beta regulates tubular epithelial-myofibroblast transdifferentiation in vitro. *Kidney International* 1999; **56**: 1455-67.

19. Goumenos DS, Tsakas S, El Nahas AM *et al.* Transforming growth factor-beta (1) in the kidney and urine of patients with glomerular disease and proteinuria. *Nephrol Dial Transplant* 2002; **17**: 2145-52.

20. Shen N, Lin H, Wu T *et al.* Inhibition of TGF-beta1-receptor posttranslational core fucosylation attenuates rat renal interstitial fibrosis. *Kidney International* 2013; **84**: 64-77.

21. Wang B, Jha JC, Hagiwara S *et al.* Transforming growth factor-beta1-mediated renal fibrosis is dependent on the regulation of transforming growth factor receptor 1 expression by let-7b. *Kidney International* 2014; **85**: 352-61.

22. Kempf T, Wollert KC. Growth-differentiation factor-15 in heart failure. *Heart Fail Clin* 2009; **5**: 537-47.

23. Lajer M, Jorsal A, Tarnow L *et al.* Plasma growth differentiation factor-15 independently predicts all-cause and cardiovascular mortality as well as deterioration of kidney function in type 1 diabetic patients with nephropathy. *Diabetes care* 2010; **33**: 1567-72.

24. Ho JE, Hwang SJ, Wollert KC *et al.* Biomarkers of cardiovascular stress and incident chronic kidney disease. *Clin Chem* 2013; **59**: 1613-20.

25. Hellemons ME, Mazagova M, Gansevoort RT *et al.* Growth-differentiation factor 15 predicts worsening of albuminuria in patients with type 2 diabetes. *Diabetes care* 2012; **35**: 2340-6.

26. Fassett RG, Venuthurupalli SK, Gobe GC *et al.* Biomarkers in chronic kidney disease: a review. *Kidney International* 2011 **80**(8): 806-821.

27. Atlas SA. The renin-angiotensin aldosterone system: pathophysiological role and pharmacologic inhibition. *J Manag Care Pharm* 2007; **13** (8 Suppl B): 9-20.

28. Kawarazaki W, M. Nagase, Yoshida S *et al.* Angiotensin II- and salt-induced kidney injury through Rac1-mediated mineralocorticoid receptor activation. *J Am Soc Nephrol* 2012; **23**: 997-1007.

29. Imig J, Inscho EW. Adaptations of the renal microcirculation to hypertension. *Microcirculation* 2002; **9**: 315-28.

30. Abbate M, Zoja C, Remuzzi G. How does proteinuria cause progressive renal damage? *J Am Soc Nephrol* 2006; **17**: 2974-84.

31. Ikeda K, Nara Y, Yamori Y. Indirect systolic and mean blood pressure determination by a new tail cuff method in spontaneously hypertensive rats. *Lab Anim* 1991; **25**:26-9.

32. Qi Z, Breyer MD. Measurement of glomerular filtration rate in conscious mice. *Methods Mol Biol* 2009; **466**: 61-72.

33. Ding W, Li Z, Shen X *et al.* Reversal of Isoflurane-Induced Depression of Myocardial Contraction by Nitroxyl via Myofilament Sensitization to Ca^{2+}. *J Pharmacol Exp Ther* 2011; **339**: 825-831.

34. Del Turco S, Gaggini M, Daniele G *et al.* Insulin resistance and endothelial dysfunction: a mutual relationship in cardiometabolic risk. *Curr Pharm Design* 2013; **19**:2420-31.

35. Reffelmann T, Kloner RA. Transthoracic echocardiography in rats. Evaluation of commonly used indices of left ventricular dimensions, contractile performance, and hypertrophy in a genetic model of hypertrophic heart failure (SHHF-Mcc-facp-Rats) in comparison with Wistar rats during aging. *Basic Res Cardiol* 2003; **98**: 275-284.

36. Smyth, GK. Limma: linear models for microarray data. In: Bioinformatics and Computational Biology Solutions using R and Bioconductor, R. Gentleman, V. Carey, S. Dudoit, R. Irizarry, W. Huber (eds.), Springer, New York, pages 397-420.

37. Wu D and Smyth GK. Camera: a competitive gene set test accounting for inter-gene correlation. *Nucleic acids research* 2012 **40**(17): e133.

38. Benjamini, Y and Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society* 1995; Series B (Methodological) **57**(1): 289-300.

39. Sharma S, Sirin Y, Susztak K *et al.* The story of Notch and chronic kidney disease. *Curr Opin Nephrol Hy* 2011; **20**: 56-61.

40. Brown NJ. Contribution of aldosterone to cardiovascular and renal inflammation and fibrosis. *Nat Rev Nephrol* 2013; **9**: 459-69.

41. Ritz E, Tomaschitz A. Aldosterone and kidney: a rapidly moving frontier (an update). *Nephrol Dial Transplant* 2013; **0**:1-9.

42. Hargovan M, Ferro A. Aldosterone synthase inhibitors in hypertension: current status and future possibilities. *JRSM Cardiovasc Dis* 2014; **3**.

43. Ichikawa D, Kamijo-Ikemori A, Sugaya T *et al.* Renoprotective effect of renal liver-type fatty acid binding protein and angiotensin II type 1a receptor loss in renal injury caused by RAS activation. *Am J Physiol Renal Physiol* 2014; **306**: F655-F663.

44. McKelvie RS, Yusuf S, Pericak D *et al.* Comparison of candesartan, enalapril, and their combination in congestive heart failure: randomized evaluation of strategies for left ventricular dysfunction (RESOLVD) pilot study. The RESOLVD Pilot Study Investigators. *Circulation* 1999; **100**: 1056-64.

45. Barrera-Chimal J, Perez-Villalva R, Rodríguez-Romo R *et al.* Spironolactone prevents chronic kidney disease caused by ischemic acute kidney injury. *Kidney International* 2013; **83**: 93-103.

46. Chen H, Sun F, Zhong X *et al.* Eplerenone-mediated aldosterone blockade prevents renal fibrosis by reducing renal inflammation, interstitial cell proliferation and oxidative stress. *Kidney Blood Press* R 2013; **37**: 557-66.

47. Williams JS. Evolving research in nongenomic actions of aldosterone. *Curr Opin Endocrinol Diabetes Obes* 2013**; 20**(3): 198-203.

48. Nistala R, Wei Y, Sowers JR *et al.* RAAS-mediated Redox effects in Chronic Kidney Disease. *Transl Res* 2009; **153**(3):102-113.

49. Fiorini G, Scotti LA, Parmigiani ML *et al.* An echocardiographic study of left ventricular diastolic function in patients with diabetes mellitus type 2. *G Ital Cardiol* 2004; **25**: 17-25.

50. Stahrenberg R, Edelmann F, Mende M *et al.* Association of glucose metabolism with diastolic function along the diabetic continuum. *Diabetologia* 2010; **53**: 1331-1340.

Figures and tables

Figure 1 **(A)- MAP measured non-invasively in ZSF1 rats along 6 weeks of treatment, from 26W to 32W of age, Data are expressed as mean ± SEM; n=5-8. (B)- Impact of 6 weeks of AngII infusion or Lisinopril treatment on RAAS hormones levels and renin activity measured in plasma of 32W-old ZSF1 rats. Data are expressed as mean ± SEM; n=7-10. (C)- Zona glomerulosa section of a representative adrenal gland of a Vehicle-treated ZSF1 rat (left) and AngII infused ZSF1 rat (right) at 32W of age (HE staining).**

Figure 2 **(A)- GFR values measured in 32W-old ZSF1 rats, after 6 weeks of treatment. Data are expressed as mean ± SEM; n=8-10. (B)- Changes in Albuminuria and Creatinine clearance of ZSF1 rats along treatment. Data are expressed as mean ± SEM; n=8-10. (C)- IHC scoring of main representative kidney structures after 6 weeks of treatment. Values are mean ± maximum and minimum findings; n=8-10. (D)- Ejection fraction (calculated) and left ventricular mass (measured) in ZSF1 rats after 6 weeks of treatment. Data are expressed as mean ± SEM; n=8.**

Figure 3 **Gene expression analysis and gene-set enrichment from ZSF1 rat kidney. AngII and Lisinopril regulate gene expression of pathways involved in inflammation and fibrosis reversely in kidney. (A) Global gene expression patterns of whole kidney from ZSF1 rats treated with vehicle, AngII and lisinopril, respectively, revealed by co-inertia analysis. Treatments with two compounds caused opposite effects on the transcriptomic level. (B-E) Gene-set enrichment analysis revealed consistent up- and down-regulation of genes in several inflammation- and fibrosis-associated biological networks Notch, TGF-beta, cell cycle, and glucocorticoid pathway (FDR<0.01). Kidney samples are arranged in columns and color-coded by treatment groups: vehicle (red), angiotensin II (blue), and lisinopril (green). Most genes in the four pathways are consistently up-regulated by Angiotensin II and down-regulated by lisinopril.**

Figure 4 **(A)- Impact of 6 weeks treatment on plasma Aldosterone levels in 32 week-old ZSF1 rats. Data are expressed as mean ± SEM; n=6-8. (B)- GFR values of 32 week-old ZSF1 rats, after 6 weeks of treatment. Data are expressed as mean ± SEM; n=5-8. (C)- Changes in Albuminuria and Creatinine clearance of ZSF1 rats along treatment. Data are expressed as mean ± SEM; n=5-8. (D)- IHC scoring of main representative kidney structures after 6 weeks of treatment; Values are mean ± maximum and minimum findings; n=6-8. (E)- Ejection fraction and left ventricular mass in ZSF1 rats after 6 weeks of treatment. Data are expressed as mean ± SEM; n=6-8.**

Figure 5 **Gene expression regulation by (ASI) in kidney, heart and adrenal glands.(A) Global expression patterns revealed by co-inertia analysis. (B) Relative gene expression levels of the four pathways that are significantly regulated by AngII in our model (figure 3). Gene expression levels induced by AngII, AngII+Eplerenone, and AngII+ASI are compared respectively with vehicle by one-way ANOVA.**

Table 1 **Urine kidney BM evolution in ZSF1 rats along treatment. Data are expressed as mean ± SEM; n=8-10.**

Table 2 **Urine kidney BM evolution in ZSF1 rats along treatment. Data are expressed as mean ± SEM; n=5-8.**

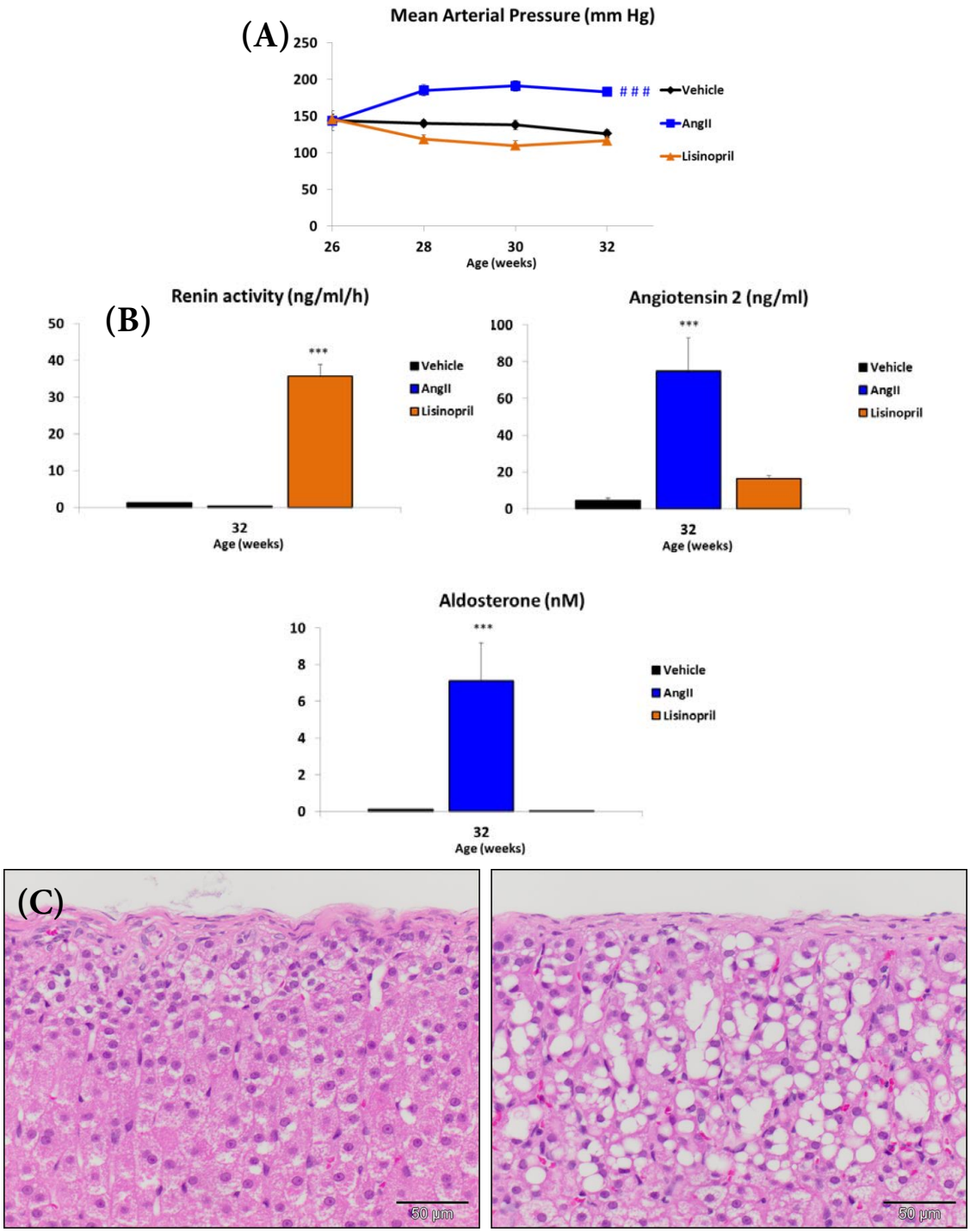

(A)
Mean Arterial Pressure (mm Hg)
250
200
150
100
50
0
26
28
30
32
Age (weeks)
# # #
Vehicle
AngII
Lisinopril
(B)
Renin activity (ng/ml/h)
50
40
30
20
10
0
***
32
Age (weeks)
Vehicle
AngII
Lisinopril
Angiotensin 2 (ng/ml)
100
80
60
40
20
0
***
32
Age (weeks)
Vehicle
AngII
Lisinopril
Aldosterone (nM)
10
8
6
4
2
0
***
32
Age (weeks)
Vehicle
AngII
Lisinopril
(C)
50 µm
50 µm

Figure 1

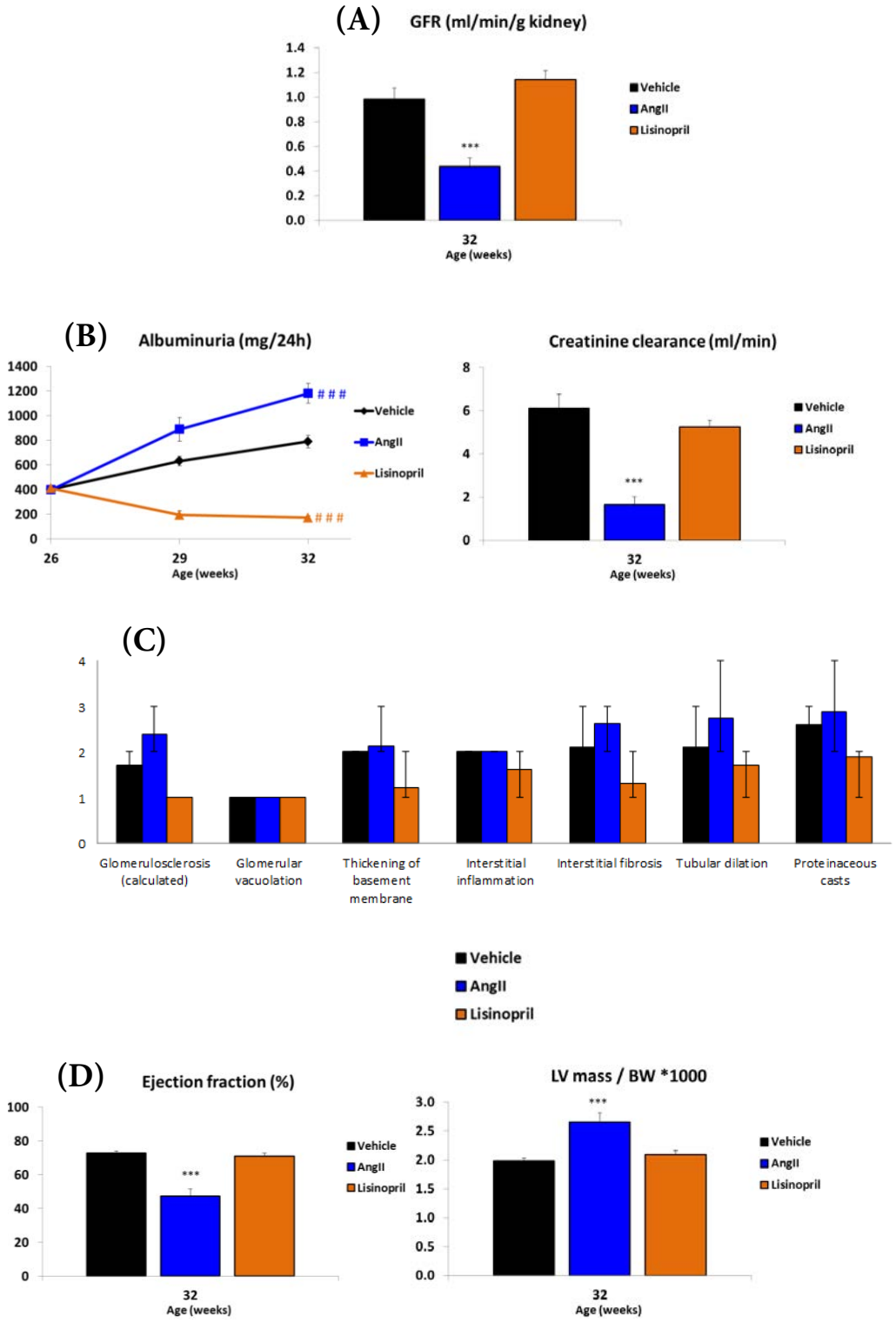

(A)
GFR (ml/min/g kidney)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
32
Age (weeks)
Vehicle
AngII
Lisinopril
(B)
Albuminuria (mg/24h)
1400
1200
1000
800
600
400
200
0
26
29
32
Age (weeks)
# # #
# # #
Vehicle
AngII
Lisinopril
Creatinine clearance (ml/min)
8
6
4
2
0
***
32
Age (weeks)
Vehicle
AngII
Lisinopril
(C)
4
3
2
1
0
Glomerulosclerosis (calculated)
Glomerular vacuolation
Thickening of basement membrane
Interstitial inflammation
Interstitial fibrosis
Tubular dilation
Proteinaceous casts
Vehicle
AngII
Lisinopril
(D)
Ejection fraction (%)
100
80
60
40
20
0
***
32
Age (weeks)
Vehicle
AngII
Lisinopril
LV mass / BW *1000
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
32
Age (weeks)
Vehicle
AngII
Lisinopril

Figure 2

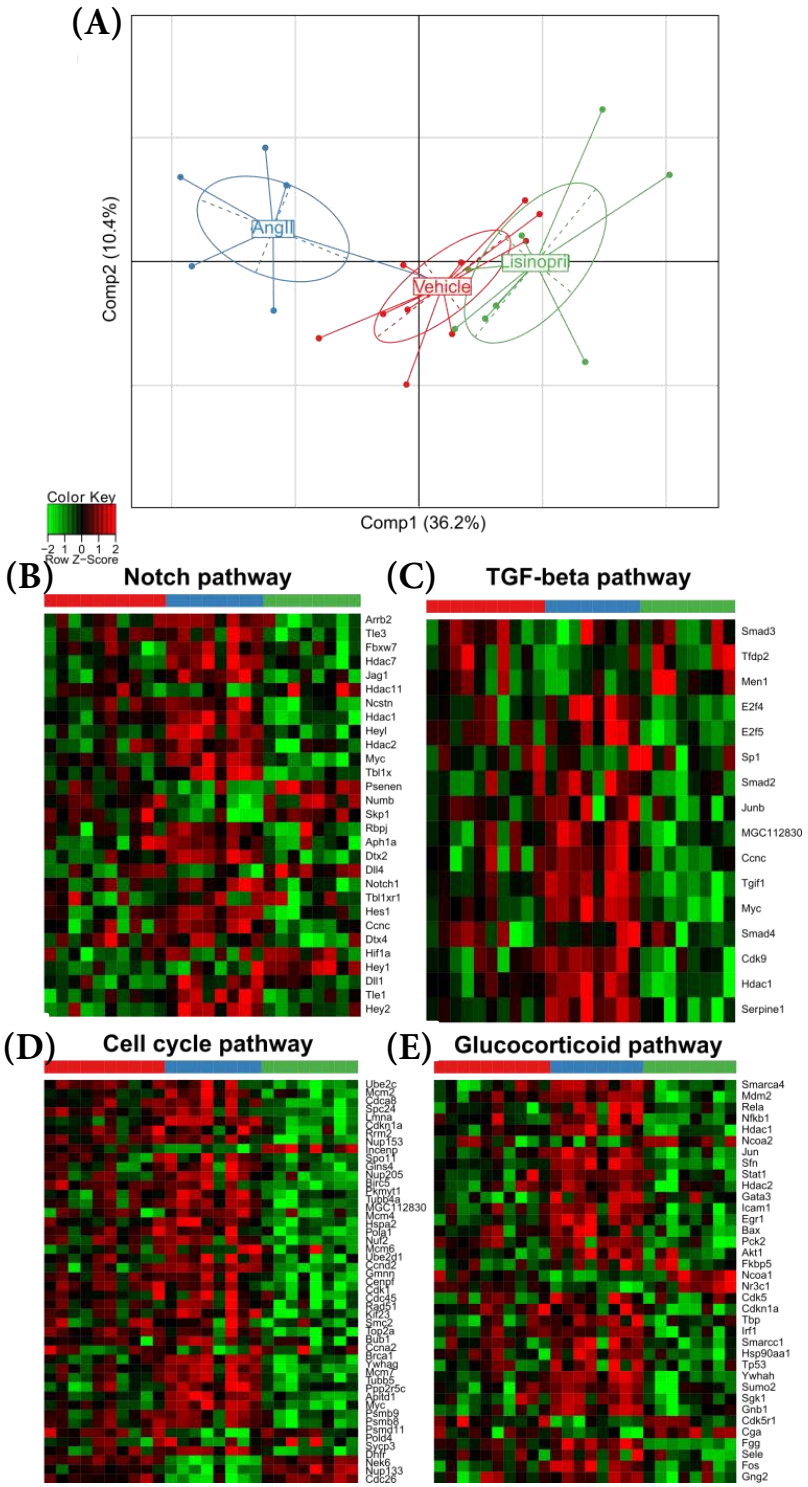
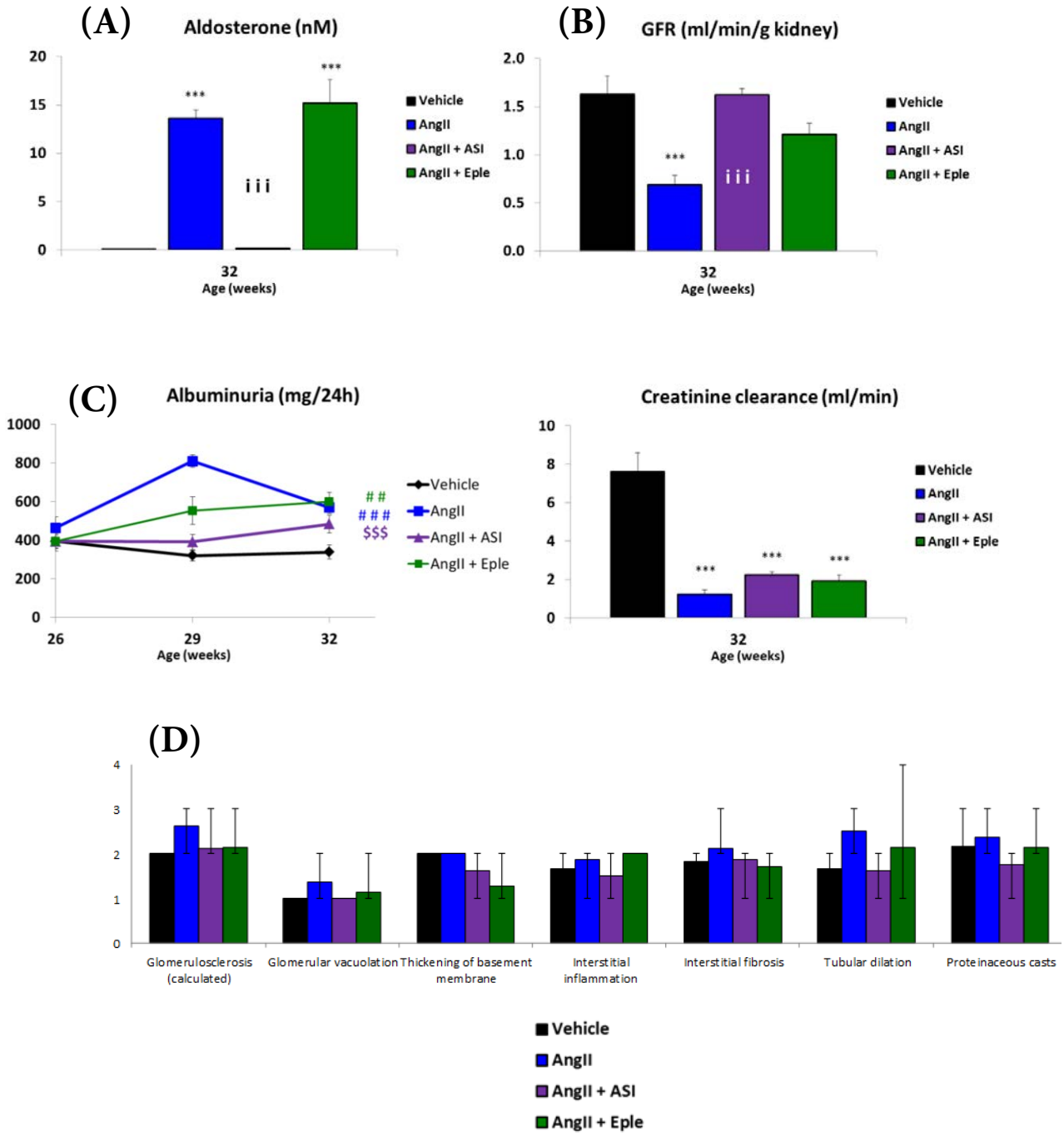

(A)
AngII
Vehicle
Lisinopril
Comp2 (10.4%)
Comp1 (36.2%)
Color Key
−2 −1 0 1 2
Row Z−Score
(B)
Notch pathway
Arrb2
Tle3
Fbxw7
Hdac7
Jag1
Hdac11
Ncstn
Hdac1
Heyl
Hdac2
Myc
Tbl1x
Psenen
Numb
Skp1
Rbpj
Aph1a
Dtx2
Dll4
Notch1
Tbl1xr1
Hes1
Ccnc
Dtx4
Hif1a
Hey1
Dll1
Tle1
Hey2
(C)
TGF-beta pathway
Smad3
Tfdp2
Men1
E2f4
E2f5
Sp1
Smad2
Junb
MGC112830
Ccnc
Tgif1
Myc
Smad4
Cdk9
Hdac1
Serpine1
(D)
Cell cycle pathway
Ube2c
Mcm2
Cdca8
Spc24
Lmna
Cdkn1a
Krm2
Nup153
Incenp
Spo11
Gins4
Nup205
Bir5
Pkmyt1
Tubb4a
MGC112830
Mcm4
Hspa2
Pola1
Nuf2
Mcm6
Ube2g1
Ccnd2
Gmnn
Cenpf
Cdk1
Cdc45
Rad51
Kif23
Smc2
Top2a
Bub1
Ccna2
Brca1
Ywhag
Mcm7
Tubb5
Ppp2r5c
Apitd1
Myc
Psmb9
Psmb8
Psmd11
Pold4
Sycp3
Mtf1
Nek6
Nup133
Cdc26
(E)
Glucocorticoid pathway
Smarca4
Mdm2
Rela
Nfkb1
Hdac1
Ncoa2
Jun
Sfn
Stat1
Hdac2
Gata3
Icam1
Egr1
Bax
Pck2
Akt1
Fkbp5
Ncoa1
Nr3c1
Cdk5
Cdkn1a
Tbp
Irf1
Smarcc1
Hsp90aa1
Tp53
Ywhah
Sumo2
Sgk1
Gnb1
Cdk5r1
Cga
Fgg
Sele
Fos
Gng2

Figure 3


(A)
Aldosterone (nM)
20
15
10
5
0
***
***
i i i
32
Age (weeks)
Vehicle
AngII
AngII + ASI
AngII + Eple
(B)
GFR (ml/min/g kidney)
2.0
1.5
1.0
0.5
0.0
***
i i i
32
Age (weeks)
Vehicle
AngII
AngII + ASI
AngII + Eple
(C)
Albuminuria (mg/24h)
1000
800
600
400
200
0
26
29
32
Age (weeks)
# #
# # #
$$$
Vehicle
AngII
AngII + ASI
AngII + Eple
Creatinine clearance (ml/min)
10
8
6
4
2
0
***
***
***
32
Age (weeks)
Vehicle
AngII
AngII + ASI
AngII + Eple
(D)
4
3
2
1
0
Glomerulosclerosis (calculated)
Glomerular vacuolation
Thickening of basement membrane
Interstitial inflammation
Interstitial fibrosis
Tubular dilation
Proteinaceous casts
Vehicle
AngII
AngII + ASI
AngII + Eple

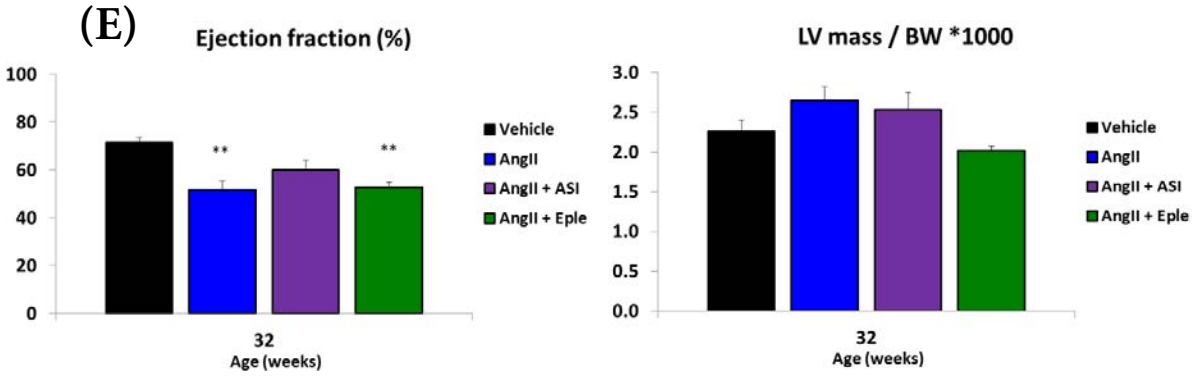
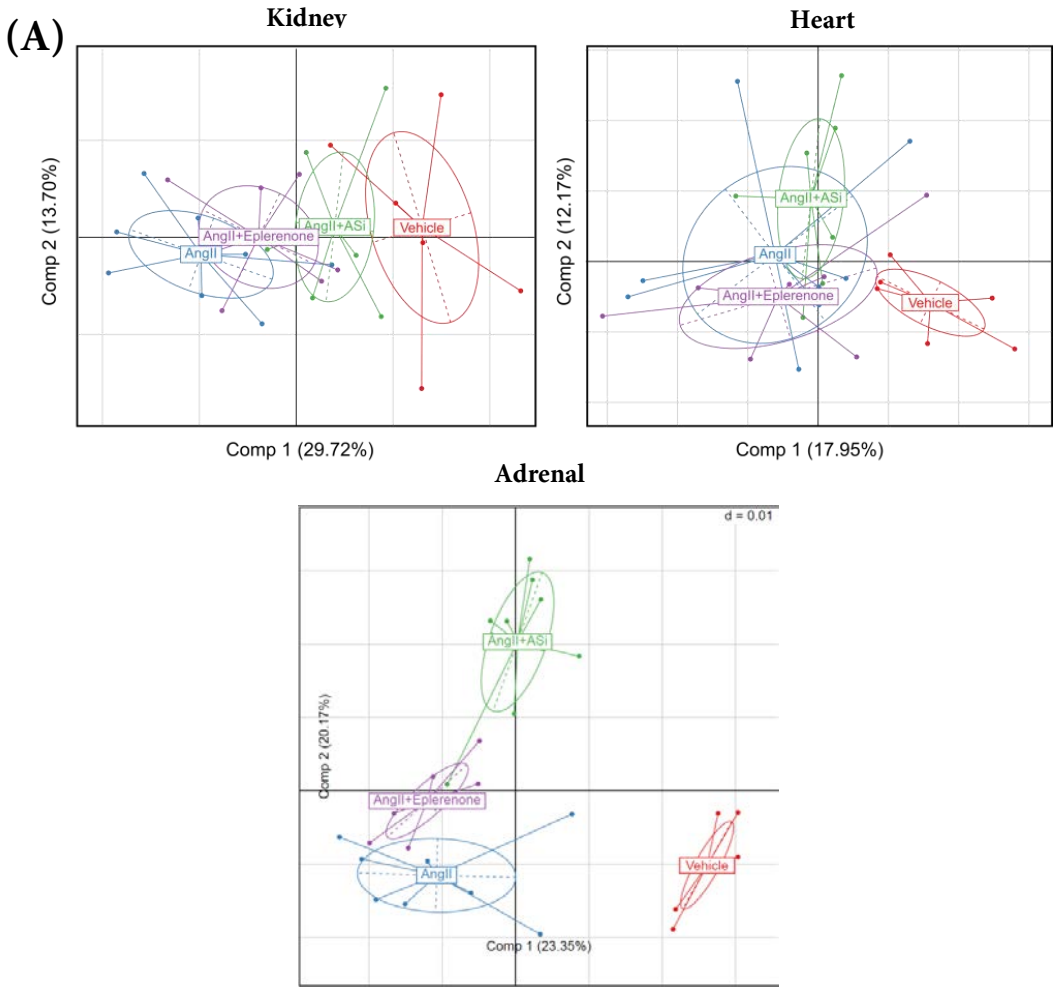

(E)
Ejection fraction (%)
100
80
60
40
20
0
**
**
32
Age (weeks)
Vehicle
AngII
AngII + ASI
AngII + Eple
LV mass / BW *1000
3.0
2.5
2.0
1.5
1.0
0.5
0.0
32
Age (weeks)
Vehicle
AngII
AngII + ASI
AngII + Eple

Figure 4


(A)
Kidney
Comp 2 (13.70%)
Comp 1 (29.72%)
AngII+Eplerenone
AngII
AngII+ASi
Vehicle
Heart
Comp 2 (12.17%)
Comp 1 (17.95%)
AngII+ASi
AngII
AngII+Eplerenone
Vehicle
Adrenal
d = 0.01
Comp 2 (20.17%)
Comp 1 (23.35%)
AngII+ASi
AngII+Eplerenone
AngII
Vehicle

(B)

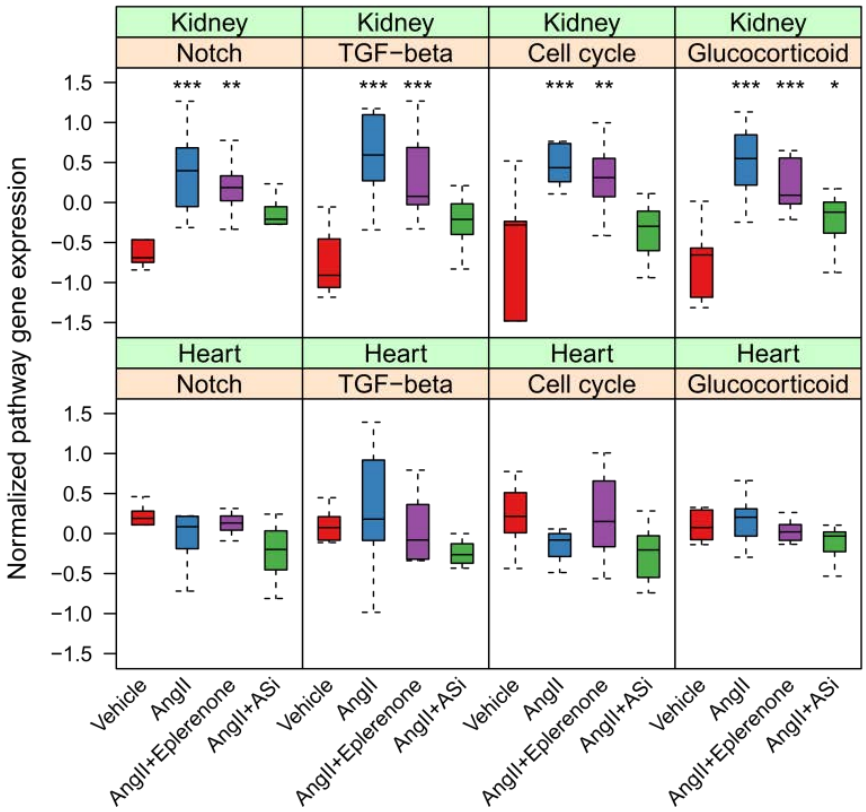

Kidney
Notch
Kidney
TGF−beta
Kidney
Cell cycle
Kidney
Glucocorticoid
Heart
Notch
Heart
TGF−beta
Heart
Cell cycle
Heart
Glucocorticoid
Normalized pathway gene expression
1.5
1.0
0.5
0.0
−0.5
−1.0
−1.5
***
**
***
***
***
**
***
***
*
Vehicle
AngII
AngII+Eplerenone
AngII+ASi

Figure 5

Table 1

	Urine Biomarkers														
	TGF-β1 (ng/24h)			β2Microglobulin (mg/24h)			Cystatin C (µg/24h)			KIM-1 (ng/24h)			GDF15 (ng/24h)		
Age (weeks)	26	29	32	26	29	32	26	29	32	26	29	32	26	29	32
Vehicle	2.1 ± 0.2	3.0 ± 0.3	2.6 ± 0.2	12 ± 1	18 ± 1	25 ± 2	46 ± 4	61 ± 4	88 ± 5	86 ± 7	127 ± 10	127 ± 10	16 ± 2	30 ± 3	59 ± 12
AngII	1.9 ± 0.2	6.4 ± 0.7	6.5 ± 0.6 # # #	11 ± 1	22 ± 2	75 ± 9 # # #	46 ± 3	98 ± 11	198 ± 15 # # #	82 ± 6	237 ± 25	297 ± 15 # # #	16 ± 2	150 ± 25	236 ± 17 # # #
Lisinopril	1.8 ± 0.3	1.9 ± 0.2	1.1 ± 0.1 # #	12 ± 1	9 ± 2	12 ± 1 # #	46 ± 4	28 ± 4	38 ± 4 # # #	74 ± 9	61 ± 5	64 ± 4 # # #	17 ± 3	14 ± 4	24 ± 7

Table 2

	Urine Biomarkers														
	TGF-β1 (ng/24h)			β2Microglobulin (mg/24h)			Cystatin C (µg/24h)			KIM-1 (ng/24h)			GDF15 (ng/24h)		
Age (weeks)	26	29	32	26	29	32	26	29	32	26	29	32	26	29	32
Vehicle	1.0 ± 0.4	1.8±0.3	2.6±0.4	30±5	14±2	32±4	60±8	67±10	111±14	80±11	88±14	104±18	10±2	23±7	34±6
AngII	1.8 ±0.4	6.4±0.5	8.6±0.7 # # #	26±5	28±4	88±6 # # #	65±10	175±24	264±26 # # #	85±12	324±29	242±19 # # #	14±2	138±14	77±6 # # #
AngII + ASI	1.7±0.5	2.9±0.3	5.0±0.4 # $ $ $	29±2	14±1	58±8 $	63±4	78±8	172±21 $ $	78±6	127±21	190±23 # $ $ $	9±1	82±7	66±7 # # # $ $
AngII + Eple	1.2±0.3	4.2±0.7	7.3±0.5 # # # $	28±4	21±4	73±14 # #	59±7	124±24	256±29 # # #	84±7	179±32	213±15 # # $ $	10±2	84±19	77±10 # # # $

Discussion – Conclusion

Dans une étude pilote, nous avions tout d'abord testé d'infuser de l'AngII chez des animaux plus jeunes, soit à 12 semaines, durant 6 semaines. Il s'était avéré que les dégradations cardio-rénales n'étaient pas significativement plus importantes par rapport à celles des rats ZSF1 naïfs. Nous avons donc pensé qu'il était nécessaire que des altérations soient déjà présentes au niveau du coeur et du rein, pour que le stress hémodynamique et hormonal induit par l'infusion d'AngII ait un impact néfaste sur ces organes. Nous savions depuis l'étude longitudinale de la fonction cardiaque chez le rat ZSF1 (cf. Publication 1 p.95) que la fonction diastolique était déjà dégradée à 18 semaines, et que des changements structuraux visibles avaient lieu à partir de 24 semaines. Pour avoir une chance d'exacerber la dysfonction cardiaque, nous avons donc choisi cet âge. Sachant que sans intervention le rat ZSF1 développe une néphropathie vers 50 semaines, nous avons de plus pensé que l'infusion d'AngII devrait engendrer une baisse prématurée de la fonction rénale, entre 26 et 32 semaines.

Dans la première partie de l'étude, nous avons montré que l'AngII induisait une forte hypertension, que les taux d'aldostérone plasmatiques étaient très significativement augmentés, et que les fonctions cardiaque et rénale étaient rapidement et assez fortement altérées. En effet, le taux de filtration glomérulaire a été fortement baissé et les biomarqueurs ont montré une dégradation de la structure et de la fonction rénale. De plus, la fonction systolique a été baissée de 35 % alors que chez les rats ZSF1 naïfs, nous avons vu que la fraction d'éjection était toujours préservée à 45 semaines (cf. Publication 1 p.93). Nous sommes donc parvenus à amplifier le développement des dysfonctions cardio-rénales chez le rat ZSF1, et à les induire dans un temps plus court. De plus l'analyse d'expression génique a mis en évidence que ces altérations fonctionnelles au niveau rénal étaient en association avec l'activation des voies de signalisation Notch, TGF-β, la voie des Glucocorticoïdes et du Cycle Cellulaire.

La voie Notch n'est normalement impliquée que lors de l'organogénèse du rein, puis peut être réactivée notamment lors de l'apparition d'une fibrose (Sharma *et al.* 2011). La voie des

glucocorticoïdes est impliquée dans la pathogénèse de différentes maladies associées à la néphropathie, comme l'hypertension, le diabète, et la résistance à l'insuline (Slee 2012). De plus, TGF-β1 est connu pour être impliqué dans les maladies tubulo-interstitielles, et glomérulaires (Loeffler and Wolf 2014). Nos résultats indiquent que le modèle cardio-rénal ZSF-AngII devrait pouvoir permettre de tester l'efficacité protectrice de l'inhibiteur de l'aldostérone synthase (ASi) développé en interne, sur le rein et le coeur.

Il a été suggéré qu'un inhibiteur sélectif de l'aldostérone synthase pourrait montrer une meilleure protection rénale et cardiaque dans un contexte de syndrome métabolique qu'un antagoniste des récepteurs aux minéralocorticoïdes (Hellemons *et al.* 2012). Nous avons donc testé cette hypothèse dans notre modèle ZSF-AngII, en reproduisant le synopsis de la première partie de l'étude, et testé l'efficacité de l'éplérénone face à un ASi sélectif. Comme précédemment, l'hypertension a été exacerbée, le taux d'aldostérone circulant fortement augmenté, les fonctions cardiaque et rénale impactées par l'infusion d'AngII.

Dans ce modèle cardio-rénal, l'ASi a induit une protection plus importante de la function rénale, comparée à l'éplérénone, malgré une hypertension élevée et comparable. Par consequent, cela confirme le fait que l'hypertension n'est pas le seul déclencheur des altérations rénales, mais que l'aldostérone y tient un rôle important. Ceci est corroboré par le fait que la meilleure protection rénale a été obtenue lorsque les taux d'aldostérone étaient les plus bas, c'est-à-dire avec l'ASi. Cependant, la détérioration de la fonction cardiaque induite par l'AngII n'a pas été prévenue par l'ASi ni par l'éplérénone. Ceci suggère que la durée du traitement était peut-être insuffisante, et/ou que d'autres mécanismes sont impliqués. La superiorité de l'efficacité de l'ASi sur l'éplérénone est également à mettre en relation avec les profils d'expression géniques, en particulier au niveau du rein.

Nous avons donc tenté d'améliorer le modèle ZSF1 en infusant de l'AngII pour évaluer sa capacité à mimer le syndrome métabolique et les comorbidities rénales et cardiaques associées, retrouvées chez l'Homme. Il s'est avéré que le modèle de rat ZSF1-AngII est le premier modèle montrant une forte similarité avec l'Homme, avec une dysfonction cardio-rénale dans un contexte de syndrome

métabolique, pouvant se montrer très utile pour le développement de nouvelles thérapies. Enfin, l'utilisation de ce modèle nous a permis de montrer que la protection rénale engendrée par un traitement avec un ASi sélectif était meilleure que celle offerte par l'éplérénone, composé de référence.

Discussion – Conclusion

In a previous study we first tried to infuse AngII in younger animals (12 weeks-old) for 6 weeks but only trends in worsening organ structures and functions were seen. We thus hypothesized that pushing the RAAS when the defects are starting to appear should have a better impact on exacerbation of cardiorenal impairment. As we had already evaluated cardiac function along aging in the first experimental work (cf. Publication 1 p.95), we knew that diastolic function was impaired from 18 weeks of age, and histology showed structural changes from 24 weeks of age. In order to worsen cardiac function, we had to start the AngII infusion after that age. Knowing that these animals naturally developed chronic kidney disease at about 50 weeks, we made the assumption that pushing the RAAS from 26 to 32 weeks should lead to renal impairment as well.

In study one, we show that chronic subcutaneous AngII infusion in 26 weeks-old male ZSF1 rats (for six weeks) led to significant increase in blood pressure. Moreover, significant increase in circulating aldosterone and significantly impaired cardiac and renal functions were achieved, to an extent that mimics diabetic nephropathy and associated co-morbidities in humans. Glomerular filtration rate and biomarkers were significantly worsened with a 6-weeks AngII infusion only, which shows that AngII strongly reduced the time frame needed to observe renal failure (32 weeks of age versus more than 50 weeks). Regarding cardiac function, systolic function dropped by 35%, while cardiac function was almost kept unchanged in ZSF1 rats until one year of age. The significant reduction of time-frame to achieve worsening of kidney function in the ZSF1 rat model and the induction of cardiac dysfunction indicated that this model can become pivotal for supporting optimal drug discovery. Our data showed as well that in the ZSF1-AngII model, AngII led to faster and greater renal damages at the kidney

level in association with activation of TGF-β, Notch, Glucocorticoid and Cell cycle pathways. Notch pathway has been shown to be important during kidney development, and then silenced when the development is complete. It has also recently been shown that this pathway is reactivated in several renal disorders implying fibrosis (Sharma *et al.* 2011), and that notch pathway activation seems not to be driven by AngII (Lavoz *et al.* 2012). Glucocorticoid activities are known to be involved in the pathogenesis of a number of diseases associated to chronic kidney disease, like hypertension, diabetes and insulin resistance, among others *via* the mineralocorticoid receptor (Slee 2012). Moreover, TGF-β1 is known to participate in the pathophysiology of glomerular and tubulointerstitial diseases (Loeffler and Wolf 2014). These results indicate that this new cardio-model ZSF1-AngII should be suitable to test efficacy of our in-house aldosterone synthase inhibitor (ASi) on kidney and heart protection.

It was suggested that a selective ASi could better protect targeted organs during diabetic nephropathy than MRa (Hellemons *et al.* 2012). We wanted to test this hypothesis in our model. To do so, we reproduced study one with infusion of AngII from 26 weeks to 32 weeks of age in ZSF1 rats, and added two treatments given as food admix: the in-house ASi and eplerenone. We monitored renal structure and function, as well as cardiac structure and function, and selected biomarkers for both cardiac and renal function.

Like in the previous experiment, aldosterone and blood pressure were significantly increased by AngII, glomerular filtration rate drastically dropped, kidney structure and biomarkers were worsened, as well as cardiac function.

In this cardio-renal model, ASi induced strong protection of renal structure and function as shown by the restored glomerular filtration rate. Kidney protection induced by ASi was superior compared to eplerenone, while mean arterial pressure values were similarly elevated in both studies. Consequently, as expected it confirms that hypertension is not the only trigger of renal alterations but

that aldosterone plays a central role in kidney dysfunction. That is confirmed by the blunt of aldosterone production by ASi as compared to eplerenone that did not change aldosterone levels.

Deterioration of cardiac function induced by AngII was neither prevented by ASi nor by eplerenone, suggesting either a too short treatment duration (6 weeks) or that additional pathways are involved. Only a trend in protecting cardiac function was dipayed by ASi. The overall superior efficacy of ASi on eplerenone was confirmed by gene expression profiles particularly in the kidney.

We performed selected experimental interventions in the new model (ZSF1-AngII) to evaluate its capacity of capturing human type 2 diabetes pathology and associated co-morbidities. AngII-infused ZSF1 rat is the first model that displayed similar cardiac and renal dysfunction as in diabetic nephropathic patients. On top of being a model for metabolic syndrome, ZSF1-AngII rat can be seen as a model of translational diabetic nephropathy and become useful for new drug assessment and development. Finally, with this model, we showed that a novel potent selective inhibitor of aldosterone synthase is more efficacious in reverting kidney (and heart damages in a much lesser extent) in ZSF1-AngII rats than the reference compound eplerenone.

3. GENERAL DISCUSSION – CONCLUSION

The main focus of our Cardiovascular and Metabolism disease therapeutic area was to find and develop molecules that are efficacious on human type 2 diabetes and metabolic syndrome. In the *in vivo* department, our aim was first to demonstrate efficacy in selected diabetic rodent models, such as db/db mice, or ZDF rats. Then this focus evolved towards treating the main comorbidities associated to type 2 diabetes in rodents, which implied to evaluate the capacity of compounds to maintain and/or improve kidney and cardiac functions. As a result, our focus evolved towards type 2 diabetes, hypertension, obesity, insulin resistance, inflammation, all features that have been associated to the term of Metabolic Syndrome. To be as predictive as possible, we needed to find translational rodent models that could help us capturing the beneficial effects of drugs (human standards of care and innovative new compounds) on cardiorenal protection. Our new focus being broader than focusing on insulin resistance, glycemia and HbA1c, the models we used were not suitable anymore: for example, the ZDF rat model of type 2 diabetes develops insulin resistance associated with hyperglycemia, but neither develops hypertension (Prabhakar *et al.* 2007) nor mimic the human diabetic nephropathy onset (Marsh *et al.* 2007; Pollock and Carmines 2008). This lack of translationnability with the human situation led us to compare the models that were available in house, with the ones that were described in the literature to develop a metabolic syndrome. As a result, we found out that the rodent model that could fulfill our needs was derived from the ZDF and SHHF backgrounds, the ZSF1 rat (cf. Editorial p.76). This model had been described to develop all the features of metabolic syndrome (Tofovic and Jackson 2003) and as certainly being the best available model for simulation of human diabetic nephropathy (Bilan *et al.* 2011). We wished to get a model that would develop in a time-frame compatible with the pharmaceutical industry requirements.

Only few publications described the renal function of these animals, and it was stated that this animal model develops renal failure at around 1 year of age. We monitored the cardiac function along aging, as it had not been described so far (cf. Publication 1 p.93). We found out that diastolic function was

already impaired from 18 weeks of age, but that systolic function was still maintained at 45 weeks of age.

This preliminary characterization of the ZSF1 rat model led us to test two strategies to try to worsen both renal and cardiac defects in a more suitable time-frame.

Our first attempt consisted in using renal mass reduction by UNx to induce a mechanical stress, and impair kidney function. With this process, we thought that impaired kidney function in a context of metabolic syndrome could trigger associated cardiac defects. Finally, UNx accelerated kidney structural defects apparition, and subsequent loss of kidney function, but was inefficacious in inducing cardiac impairment (cf. Publication 2 p.129, and annex 1). Despite only renal function was altered, we tested an anti-oxidant compound bardoxolone in this model to evaluate its impact on kidney protection. Our results showed that the effects of bardoxolone in UNx-ZSF1 are partially driven by body weight loss and partially by induction of Nrf2-responsive genes, and are independent of blood pressure reduction. This work didn't allow assessing the effect of bardoxolone on cardiac function. This model limitation pushed to find another alternative that combined impairment of both renal AND cardiac functions.

Consequently, we considered a different approach than renal mass reduction; we thought that pushing the RAAS could be a good alternative to exacerbate the underlying defects in ZSF1 rats' cardio-renal function, as it should induce both a hemodynamic stress on top of hyperaldosteronism. To do so, we infused AngII with subcutaneous osmotic pumps for 6 weeks in 26 week-old ZSF1 rats, and evaluated renal and cardiac functions, some selected biomarkers, as well as organ structures. As previously evoked, 26 weeks of age to start the infusion seemed optimal as we expected kidney AND renal defects, pushing the RAAS with AngII. Moreover, if we could demonstrate a worsening of cardiac and renal function already at 32 weeks of age, it would constitute a considerable gain of time already (32 weeks against 50 weeks). For these reasons we chose to start the AngII infusion at 26 weeks to get the cardiorenal status of these modified ZSF1 rat model at 32 weeks of age. With this design, we showed a significant increase in mean arterial pressure, associated with much higher

levels of plasma aldosterone levels. Systolic function dropped, associated with a left ventricle hypertrophy. Moreover, glomerular filtration rate was also significantly reduced, albuminuria was significantly increased, and associated biomarkers in the AngII-infused ZSF1 were worsened, going along with a mild worsening in kidney structure. Finally, gene expression profiling showed that in the kidney, four pathways were up regulated by AngII: Notch, TGF-β1, Cell cycle and the Glucocorticoid pathways, known to be associated with kidney disease. Having successfully reached the concomitant cardiac and renal impairments, and being in presence of high levels of aldosterone, we tested in-house ASi head to head with eplerenone in this new ZSF-AngII model. Our work showed that ASi blunted aldosterone synthesis contrary to eplerenone, which was expected. Moreover, ASi was more efficacious in protecting kidney structure and function as compared to eplerenone. ASi only showed a trend in protecting heart function whereas eplerenone did not. Finally gene expression profiling was not impacted in the heart by any treatment, whereas only ASi prevented the activation of the pathways cited above in the kidney.

To conclude, we step by step managed to improve an existing rodent model of metabolic syndrome: -infused ZSF1-AngII rat is a suitable model to evaluate the effects of drugs on cardiac and renal functions in the context of metabolic syndrome in a reasonable timeframe.

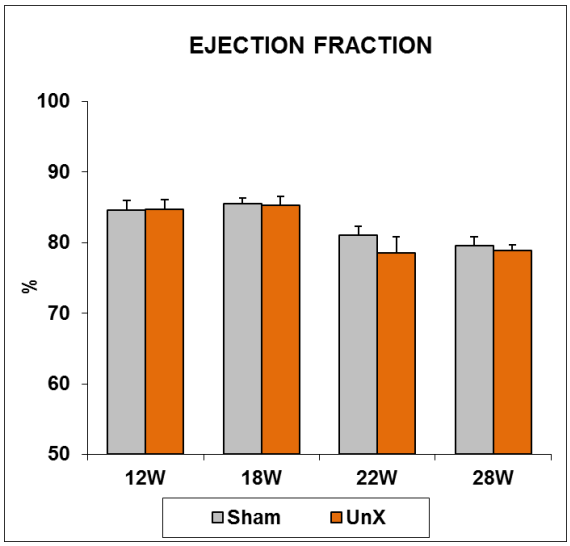
Another way to exacerbate the ZSF1 rat model could be with a high sodium intake instead of, or on top of AngII infusion. In Sprague-Dawley rats, 8% NaCl chow diet given for 5 months induced left ventricular hypertrophy, and reduction of renal function (not associated with structural changes), independently from blood pressure, which was not increased (Cordaillat *et al.* 2005). We could imagine that high salt diet should also be deleterious in the ZSF1 rat model of metabolic syndrome, as reported in the hypertensive SHR rats for example (Yu *et al.* 1998). We wanted to test in-house ASi and thus ensure that aldosterone levels would be high enough to test the proof on concept, and the effects on kidney and cardiac function, as a result, we chose to infuse AngII. The next step could

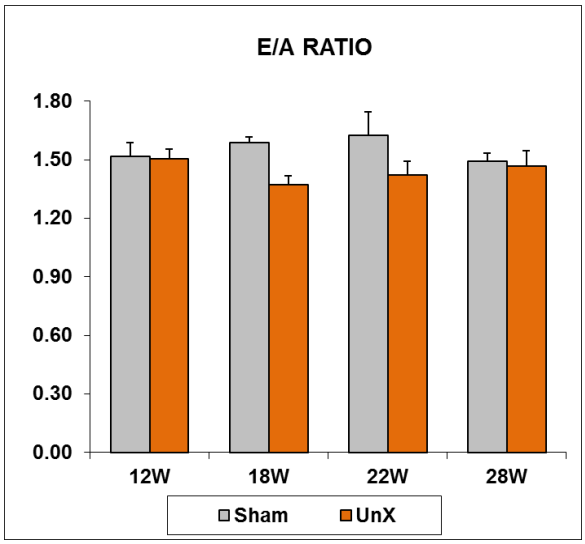
be to refine the ZSF-AngII model adding the salt component, or even skip the AngII. Indeed we showed a 35 % decrease in the systolic function with AngII infusion, which is drastic, and might not be the best case to evaluate effects of drugs on cardiac protection. It could be that a milder decrease in cardiac function could better allow depicting the protective effects of compounds on heart, and only using high salt diet could be a step in this direction. Nevertheless, would the renal defects and decrease in glomerular filtration rate enough to evaluate kidney protective effect of molecules? We really think that the way of exacerbating cardiorenal features is important, and finding the right methodology is not simple, and should offer a suitable window on both renal and cardiac dysfunction, but without impacting too much these two functions to be still able evaluating protective efficacy of standard of care and innovative compounds.

ANNEXES

<u>Annex 1:</u> Cardiac function of UNx-ZSF1 rats *vs.* Sham operated ZSF1 rats, from 12 to 28 weeks of age.

UNx did not alter cardiac function of ZSF1 rats from 12 weeks up to 28 weeks of age, as compared to Sham operated animals. Data are expressed as mean ± SEM. N=8 per group.





<u>Annex 2:</u> **Participation to publications on incretin based therapies.**

A rationally designed monomeric peptide triagonist corrects obesity and diabetes in rodents.

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We report the discovery of a new monomeric peptide that reduces body weight and diabetic complications in rodent models of obesity by acting as an agonist at three key metabolically-related peptide hormone receptors: glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP) and glucagon receptors. This triple agonist demonstrates supraphysiological potency and equally aligned constituent activities at each receptor, all without cross-reactivity at other related receptors. Such balanced unimolecular triple agonism proved superior to any existing dual coagonists and best-in-class monoagonists to reduce body weight, enhance glycemic control and reverse hepatic steatosis in relevant rodent models. Various loss-of-function models, including genetic knockout, pharmacological blockade and selective chemical knockout, confirmed contributions of each constituent activity in vivo. We demonstrate that these individual constituent

activities harmonize to govern the overall metabolic efficacy, which predominantly results from synergistic glucagon action to increase energy expenditure, GLP-1 action to reduce caloric intake and improve glucose control, and GIP action to potentiate the incretin effect and buffer against the diabetogenic effect of inherent glucagon activity. These preclinical studies suggest that, so far, this unimolecular, polypharmaceutical strategy has potential to be the most effective pharmacological approach to reversing obesity and related metabolic disorders.

Reference : Finan, B, B Yang *et al.* (2015). "A rationally designed monomeric peptide triagonist corrects obesity and diabetes in rodents." Nature Medicine **21**(1): 27-36.

Unimolecular Dual Incretins Maximize Metabolic Benefits in Rodents, Monkeys, and Humans

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We report the discovery and translational therapeutic efficacy of a peptide with potent, balanced co-agonism at both of the receptors for the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). This unimolecular dual incretin is derived from an intermixed sequence of GLP-1 and GIP, and demonstrated enhanced antihyperglycemic and insulinotropic efficacy relative to selective GLP-1 agonists. Notably, this superior efficacy translated across rodent models of obesity and diabetes, including db/db mice and ZDF rats, to primates (cynomolgus monkeys and humans). Furthermore, this co-agonist exhibited synergism in reducing fat mass in obese rodents, whereas a selective GIP agonist demonstrated negligible weight-lowering efficacy. The unimolecular dual incretins corrected two causal mechanisms of diabesity, adiposity-induced insulin resistance and pancreatic insulin deficiency, more effectively than did selective mono-

agonists. The duration of action of the unimolecular dual incretins was refined through site-specific lipidation or PEGylation to support less frequent administration. These peptides provide comparable pharmacology to the native peptides and enhanced efficacy relative to similarly modified selective GLP-1 agonists. The pharmacokinetic enhancement lessened peak drug exposure and, in combination with less dependence on GLP-1–mediated pharmacology, avoided the adverse gastrointestinal effects that typify selective GLP-1–based agonists. This discovery and validation of a balanced and high-potency dual incretin agonist enables a more physiological approach to management of diseases associated with impaired glucose tolerance.

Reference : Finan, B, T Ma *et al.* (2013). "Unimolecular Dual Incretins Maximize Metabolic Benefits in Rodents, Monkeys, and Humans." Science Translational Medicine **5**(209): 209ra151.

REFERENCES

(2002). "Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report." Circulation **106**(25): 3143-3421.

(2003). "Report of the expert committee on the diagnosis and classification of diabetes mellitus." Diabetes care **26**(1): S5-20.

ADA (2005). "Clinical Practice Recommendations 2005." Diabetes care **28**(1): S1-79.

Adler, AI, RJ Stevens, SE Manley, RW Bilous, CA Cull and RR Holman (2003). "Development and progression of nephropathy in type 2 diabetes: the United Kingdom Prospective Diabetes Study (UKPDS 64)." Kidney International **63**(1): 225-232.

Ahmed, N (2005). "Advanced glycation endproducts—role in pathology of diabetic complications." Diabetes Research and Clinical Practice **67**(1): 3-21.

Alberti, KG, RH Eckel, SM Grundy, PZ Zimmet, JI Cleeman, KA Donato, JC Fruchart, WP James, CM Loria and SC Smith, Jr. (2009). "Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity." Circulation **120**(16): 1640-1645.

Alberti, KG, P Zimmet and J Shaw (2005). "The metabolic syndrome--a new worldwide definition." Lancet **366**(9491): 1059-1062.

Alderman, MH, S Madhavan, WL Ooi, H Cohen, JE Sealey and JH Laragh (1991). "Association of the renin-sodium profile with the risk of myocardial infarction in patients with hypertension." New England Journal of Medicine **324**(16): 1098-1104.

Aleksunes, LM, MJ Goedken, CE Rockwell, J Thomale, JE Manautou and CD Klaassen (2010). "Transcriptional regulation of renal cytoprotective genes by Nrf2 and its potential use as a therapeutic target to mitigate cisplatin-induced nephrotoxicity." The Journal of pharmacology and experimental therapeutics **335**(1): 2-12.

ALLHAT (2002). "Major outcomes in high-risk hypertensive patients randomized to angiotensin-converting enzyme inhibitor or calcium channel blocker vs diuretic: The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT)." JAMA **288**(23): 2981-2997.

Alp, NJ and KM Channon (2004). "Regulation of Endothelial Nitric Oxide Synthase by Tetrahydrobiopterin in Vascular Disease." Arteriosclerosis, thrombosis, and vascular biology **24**(3): 413-420.

Anavekar, NS, DJ Gans, T Berl, RD Rohde, W Cooper, A Bhaumik, LG Hunsicker, JL Rouleau, JB Lewis, C Rosendorff, JG Porush, PL Drury, E Esmatjes, I Raz, P Vanhille, F Locatelli, S Goldhaber, EJ Lewis and MA Pfeffer (2004). "Predictors of cardiovascular events in patients with type 2 diabetic nephropathy and hypertension: a case for albuminuria." Kidney International Supplement **92**(5): S50-55.

Anderson, S, HG Rennke and BM Brenner (1986). "Therapeutic advantage of converting enzyme inhibitors in arresting progressive renal disease associated with systemic hypertension in the rat." Journal of Clinical Investigation **77**(6): 1993-2000.

Andreadis, EA, GI Tsourous, CK Tzavara, DX Georgiopoulos, PM Katsanou, GE Marakomichelakis and EJ Diamantopoulos (2007). "Metabolic Syndrome and Incident Cardiovascular Morbidity and Mortality in a Mediterranean Hypertensive Population." American journal of hypertension **20**(5): 558-564.

Appel, GB, J Radhakrishnan, MM Avram, RA DeFronzo, F Escobar-Jimenez, MM Campos, E Burgess, DA Hille, TZ Dickson, S Shahinfar and BM Brenner (2003). "Analysis of Metabolic Parameters as Predictors of Risk in the RENAAL Study." Diabetes care **26**(5): 1402-1407.

Appel, LJ, MW Brands, SR Daniels, N Karanja, PJ Elmer and FM Sacks (2006). "Dietary Approaches to Prevent and Treat Hypertension: A Scientific Statement From the American Heart Association." Hypertension **47**(2): 296-308.

Arboix, A, V Cendrós, M Besa, L García-Eroles, M Oliveres, C Targa, M Balcells, E Comes and J Massons (2008). "Trends in Risk Factors, Stroke Subtypes and Outcome." Cerebrovascular Diseases **26**(5): 509-516.

Assmann, G and JR Nofer (2003). "Atheroprotective effects of high-density lipoproteins." Annual Review of Medicine **54**: 321-341.

Association, AD (2013). "Standards of Medical Care in Diabetes—2013." Diabetes care **36**(Supplement 1): S11-S66.

Astor, BC, K Matsushita, RT Gansevoort, M van der Velde, M Woodward, AS Levey, PEd Jong and J Coresh (2011). "Lower estimated glomerular filtration rate and higher albuminuria are associated with mortality and end-stage renal disease. A collaborative meta-analysis of kidney disease population cohorts." Kidney International **79**(12): 1331-1340.

Atlas, SA (2007). "The renin-angiotensin aldosterone system: pathophysiological role and pharmacologic inhibition." Journal of Managed Care Pharmacy **13**(8 Suppl B): 9-20.

Azizi, M, L Amar and J Menard (2013). "Aldosterone synthase inhibition in humans." Nephrology Dialysis Transplantation **28**(1): 36-43.

Babio, N, M Bulló, J Basora, MA Martínez-González, J Fernández-Ballart, F Márquez-Sandoval, C Molina and J Salas-Salvadó (2009). "Adherence to the Mediterranean diet and risk of metabolic syndrome and its components." Nutrition, Metabolism and Cardiovascular Diseases **19**(8): 563-570.

Bailey, CJ and RC Turner (1996). "Metformin." The New England journal of medicine **334**(9): 574-579.

Balakumar, P and G Jagadeesh (2011). "Potential cross-talk between (pro)renin receptors and Wnt/frizzled receptors in cardiovascular and renal disorders." Hypertension research : official journal of the Japanese Society of Hypertension **34**(11): 1161-1170.

Balarajan, Y and E Villamor (2009). "Nationally Representative Surveys Show Recent Increases in the Prevalence of Overweight and Obesity among Women of Reproductive Age in Bangladesh, Nepal, and India." The Journal of nutrition **139**(11): 2139-2144.

Balkau, B and MA Charles (1999). Comment on the provisional report from the WHO consultation. European Group for the Study of Insulin Resistance (EGIR), Diabet Med. 1999 May;16(5):442-3.

Barbosa, PJ, I Lessa, N de Almeida Filho, LB Magalhaes and J Araujo (2006). "Criteria for central obesity in a Brazilian population: impact on metabolic syndrome." Arquivos Brasileiros de Cardiologia **87**(4): 407-414.

Barker, J (2012). "Conquering Metabolic Syndrome " Complementary Prescriptions Journal **26**(11).

Barkoudah, E, H Skali, H Uno, SD Solomon and MA Pfeffer (2012). "Mortality Rates in Trials of Subjects With Type 2 Diabetes." Journal of the American Heart Association **1**(1): 8-15.

Bartnik, M and F Cosentino (2009). "Dysglycaemia, cardiovascular outcome and treatment. Is the jury still out?" European Heart Journal **30**(11): 1301-1304.

Basi, S, P Fesler, A Mimran and JB Lewis (2008). "Microalbuminuria in type 2 diabetes and hypertension: a marker, treatment target, or innocent bystander?" Diabetes care **31**(2): dc08-s249.

Battiprolu, PK, TG Gillette, ZV Wang, S Lavandero and JA Hill (2010). "Diabetic Cardiomyopathy: Mechanisms and Therapeutic Targets." Drug Discovery Today: Disease Mechanisms **7**(2): e135-e143.

Battiprolu, PK, B Hojayev, N Jiang, ZV Wang, X Luo, M Iglewski, JM Shelton, RD Gerard, BA Rothermel, TG Gillette, S Lavandero and JA Hill (2012). "Metabolic stress–induced activation

of FoxO1 triggers diabetic cardiomyopathy in mice." The Journal of clinical investigation **122**(3): 1109-1118.

Beckman, JA, MA Creager and P Libby (2002). "Diabetes and atherosclerosis: epidemiology, pathophysiology, and management." JAMA **287**(19): 2570-2581.

Benardeau, A, Riboulet W, M Albertini and M Gomez (2013). "Animal models of metabolic syndrome and associated co-morbidities." Journal of Experimental and Applied Animal Science **1**(1): 152-155.

Bicket, DP (2002). "Using ACE inhibitors appropriately." American Family Physician **66**(3): 461-468.

Bidani, AK and KA Griffin (2004). "Pathophysiology of Hypertensive Renal Damage: Implications for Therapy." Hypertension **44**(5): 595-601.

Bilan, VP, EM Salah, S Bastacky, HB Jones, RM Mayers, B Zinker, SM Poucher and SP Tofovic (2011). "Diabetic nephropathy and long-term treatment effects of rosiglitazone and enalapril in obese ZSF1 rats." Journal of Endocrinology**210**(3): 293-308.

Bischoff, H (1995). "The mechanism of alpha-glucosidase inhibition in the management of diabetes." Clinical and Invesigativet Medicine **18**(4): 303-311.

Bomback, AS and PJ Klemmer (2007). "The incidence and implications of aldosterone breakthrough." Nature Clinical Practice Nephrology **3**(9): 486-492.

Bonetti, PO, LO Lerman and A Lerman (2003). "Endothelial Dysfunction: A Marker of Atherosclerotic Risk." Arteriosclerosis, thrombosis, and vascular biology **23**(2): 168-175.

Bottinger, EP and M Bitzer (2002). "TGF-beta signaling in renal disease." Journal of the American Society of Nephrology : JASN **13**(10): 2600-2610.

Boustany, CM, K Bharadwaj, A Daugherty, DR Brown, DC Randall and LA Cassis (2004). "Activation of the systemic and adipose renin-angiotensin system in rats with diet-induced obesity and hypertension." American Journal of Physiology - Regulatory Integrative and Comparative Physiology **287**(4 56-4): R943-R949.

Brenner, BM, ME Cooper, D de Zeeuw, WF Keane, WE Mitch, H-H Parving, G Remuzzi, SM Snapinn, Z Zhang and S Shahinfar (2001). "Effects of Losartan on Renal and Cardiovascular Outcomes in Patients with Type 2 Diabetes and Nephropathy." New England Journal of Medicine **345**(12): 861-869.

Briet, M and EL Schiffrin (2010). "Aldosterone: effects on the kidney and cardiovascular system." Nature reviews. Nephrology **6**(5): 261-273.

Briet, M and EL Schiffrin (2011). "The role of aldosterone in the metabolic syndrome." Current Hypertension Reports **13**(2): 163-172.

Briones, AM, A Nguyen Dinh Cat, GE Callera, A Yogi, D Burger, Y He, JW Correa, AM Gagnon, CE Gomez-Sanchez, EP Gomez-Sanchez, A Sorisky, TC Ooi, M Ruzicka, KD Burns and RM Touyz (2012). "Adipocytes produce aldosterone through calcineurin-dependent signaling pathways: implications in diabetes mellitus-associated obesity and vascular dysfunction." Hypertension **59**(5): 1069-1078.

Brouwers, O, PM Niessen, G Haenen, T Miyata, M Brownlee, CD Stehouwer, JG De Mey and CG Schalkwijk (2010). "Hyperglycaemia-induced impairment of endothelium-dependent vasorelaxation in rat mesenteric arteries is mediated by intracellular methylglyoxal levels in a pathway dependent on oxidative stress." Diabetologia **53**(5): 989-1000.

Brown, MJ (2006). "Direct renin inhibition — a new way of targeting the renin system." Journal of Renin-Angiotensin-Aldosterone System **7**(2 suppl): S7-S11.

Brown, NJ (2013). "Contribution of aldosterone to cardiovascular and renal inflammation and fibrosis." Nature reviews. Nephrology **9**(8): 459-469.

Brownlee, M, H Vlassara and A Cerami (1985). "Nonenzymatic Glycosylation Products on Collagen Covalently Trap Low-Density Lipoprotein." Diabetes **34**(9): 938-941.

Cachofeiro, V, M Goicochea, SG de Vinuesa, P Oubina, V Lahera and J Luno (2008). "Oxidative stress and inflammation, a link between chronic kidney disease and cardiovascular disease." Kidney International **74**(S111): S4-S9.

Cade, WT (2008). "Diabetes-related microvascular and macrovascular diseases in the physical therapy setting." Physical Therapy **88**(11): 1322-1335.

Calhoun, DA, WB White, H Krum, W Guo, G Bermann, A Trapani, MP Lefkowitz and J Menard (2011). "Effects of a novel aldosterone synthase inhibitor for treatment of primary hypertension: results of a randomized, double-blind, placebo- and active-controlled phase 2 trial." Circulation **124**(18): 1945-1955.

Cameron, AJ, JE Shaw and PZ Zimmet (2004). "The metabolic syndrome: prevalence in worldwide populations." Endocrinology Metabolism Clinics of North America **33**(2): 351-375.

Carey, RM and HM Siragy (2003). "Newly recognized components of the renin-angiotensin system: potential roles in cardiovascular and renal regulation." Endocrine Reviews **24**(3): 261-271.

Carlyle, M, OB Jones, JJ Kuo and JE Hall (2002). "Chronic Cardiovascular and Renal Actions of Leptin: Role of Adrenergic Activity." Hypertension **39**(2): 496-501.

Carlyle, M, OB Jones, JJ Kuo and JE Hall (2002). "Chronic cardiovascular and renal actions of leptin: Role of adrenergic activity." Hypertension **39**(2 II): 496-501.

Cassis, L (2000). "Fat cell metabolism: Insulin, fatty acids, and renin." Current Hypertension Reports **2**(2): 132-138.

Cassis, LA, VL English, K Bharadwaj and CM Boustany (2004). "Differential Effects of Local Versus Systemic Angiotensin II in the Regulation of Leptin Release from Adipocytes." Endocrinology **145**(1): 169-174.

Cecelja, M and P Chowienczyk (2012). "Role of arterial stiffness in cardiovascular disease." JRSM Cardiovascular Disease **1**(4).

Chappell, MC, A Millsted, DI Diz, KB Brosnihan and CM Ferrario (1991). "Evidence for an intrinsic angiotensin system in the canine pancreas." Journal of Hypertension **9**(8): 751-759.

Chew, EY, WT Ambrosius, MD Davis, RP Danis, S Gangaputra, CM Greven, L Hubbard, BA Esser, JF Lovato, LH Perdue, DC Goff, Jr., WC Cushman, HN Ginsberg, MB Elam, S Genuth, HC Gerstein, U Schubart and LJ Fine (2010). "Effects of medical therapies on retinopathy progression in type 2 diabetes."The New England Journal of Medicine **363**(3): 233-244.

Chiarelli, F, S Gaspari and ML Marcovecchio (2009). "Role of growth factors in diabetic kidney disease." Hormone and Metabolic Research**41**(8): 585-593.

Chiasson, JL, RG Josse, R Gomis, M Hanefeld, A Karasik and M Laakso (2003). "Acarbose treatment and the risk of cardiovascular disease and hypertension in patients with impaired glucose tolerance: the STOP-NIDDM trial." JAMA **290**(4): 486-494.

Chiasson, JL, RG Josse, JA Hunt, C Palmason, NW Rodger, SA Ross, EA Ryan, MH Tan and TM Wolever (1994). "The efficacy of acarbose in the treatment of patients with non-insulin-dependent diabetes mellitus. A multicenter controlled clinical trial." Annals of Internal Medicine **121**(12): 928-935.

Chin, M, CY Lee, JC Chuang, R Bumeister, WC Wigley, ST Sonis, KW Ward and C Meyer (2013). "Bardoxolone methyl analogs RTA 405 and dh404 are well tolerated and exhibit efficacy in rodent models of Type 2 diabetes and obesity." American journal of physiology. Renal physiology **304**(12): F1438-1446.

Chin, MP, SA Reisman, GL Bakris, M O'Grady, PG Linde, PA McCullough, D Packham, ND Vaziri, KW Ward, DG Warnock and CJ Meyer (2014). "Mechanisms Contributing to Adverse Cardiovascular Events in Patients with Type 2 Diabetes Mellitus and Stage 4 Chronic Kidney Disease Treated with Bardoxolone Methyl." American journal of nephrology **39**(6): 499-508.

Chinetti, G, JC Fruchart and B Staels (2000). "Peroxisome proliferator-activated receptors (PPARs): nuclear receptors at the crossroads between lipid metabolism and inflammation." Inflammation Research **49**(10): 497-505.

Chow, FY, DJ Nikolic-Paterson, E Ozols, RC Atkins and GH Tesch (2005). "Intercellular Adhesion Molecule-1 Deficiency Is Protective against Nephropathy in Type 2 Diabetic db/db Mice." Journal of the American Society of Nephrology **16**(6): 1711-1722.

Christmas, P (2010). "Toll-Like Receptors: Sensors that Detect Infection." Nature Education **3**(9): 85.

Chrousos, GP (2009). "Stress and disorders of the stress system." Nature Reviews Endocrinology **5**(7): 374-381.

Clyne, CD, Y Zhang, L Slutsker, JM Mathis, PC White and WE Rainey (1997). "Angiotensin II and potassium regulate human CYP11B2 transcription through common cis-elements." Molecular Endocrinology **11**(5): 638-649.

Colin, S, O Briand, V Touche, K Wouters, M Baron, F Pattou, R Hanf, A Tailleux, G Chinetti, B Staels and S Lestavel (2013). "Activation of intestinal peroxisome proliferator-activated receptor-alpha increases high-density lipoprotein production." European Heart Journal **34**(32): 2566-2574.

Collier, A, A Rumley, AG Rumley, JR Paterson, JP Leach, GDO Lowe and M Small (1992). "Free Radical Activity and Hemostatic Factors in NIDDM Patients With and Without Microalbuminuria." Diabetes **41**(8): 909-913.

Collier, CA, CR Bruce, AC Smith, G Lopaschuk and DJ Dyck (2006). "Metformin counters the insulin-induced suppression of fatty acid oxidation and stimulation of triacylglycerol storage in rodent skeletal muscle." American Journal of Physiology: Endocrinology and Metabolism **291**(1): E182-189.

Coniff, RF, JA Shapiro, TB Seaton and GA Bray (1995). "Multicenter, placebo-controlled trial comparing acarbose (BAY g 5421) with placebo, tolbutamide, and tolbutamide-plus-acarbose in non-insulin-dependent diabetes mellitus." The American journal of medicine **98**(5): 443-451.

Connell, JM and E Davies (2005). "The new biology of aldosterone." Journal of Endocrinology**186**(1): 1-20.

Cootney, RW (2001). "Ultrasound Imaging: Principles and Applications in Rodent Research." ILAR Journal **42**(3): 233-247.

Cordaillat, M, C Rugale, D Casellas, A Mimran and B Jover (2005). "Cardiorenal abnormalities associated with high sodium intake: correction by spironolactone in rats." American Journal of Physiology:l Regulatory, Integrative and Comparative Physiology**289**(4): 26.

Cosentino, F, P Francia, GG Camici, PG Pelicci, TF Luscher and M Volpe (2008). "Final common molecular pathways of aging and cardiovascular disease: role of the p66Shc protein." Arteriosclerosis, Thrombosis, and Vascular Biology **28**(4): 622-628.

Cosentino, F, K Hishikawa, ZS Katusic and TF Lüscher (1997). "High Glucose Increases Nitric Oxide Synthase Expression and Superoxide Anion Generation in Human Aortic Endothelial Cells." Circulation **96**(1): 25-28.

Coughlin, SR, L Mawdsley, JA Mugarza, PM Calverley and JP Wilding (2004). "Obstructive sleep apnoea is independently associated with an increased prevalence of metabolic syndrome." European Heart Journal **25**(9): 735-741.

Coutinho, M, HC Gerstein, Y Wang and S Yusuf (1999). "The relationship between glucose and incident cardiovascular events. A metaregression analysis of published data from 20 studies of 95,783 individuals followed for 12.4 years." Diabetes care **22**(2): 233-240.

Creager, MA, TF Lüscher, pwta of, F Cosentino and JA Beckman (2003). "Diabetes and Vascular Disease: Pathophysiology, Clinical Consequences, and Medical Therapy: Part I." Circulation **108**(12): 1527-1532.

Cuspidi, C, S Meani, V Fusi, B Severgnini, C Valerio, E Catini, G Leonetti, F Magrini and A Zanchetti (2004). "Metabolic syndrome and target organ damage in untreated essential hypertensives." Journal of Hypertension **22**(10): 1991-1998.

Danaei, G, MM Finucane, Y Lu, GM Singh, MJ Cowan, CJ Paciorek, JK Lin, F Farzadfar, Y-H Khang, GA Stevens, M Rao, MK Ali, LM Riley, CA Robinson and M Ezzati (2011). "National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2·7 million participants." The Lancet **378**(9785): 31-40.

Danser, AH (2010). "The increase in renin during renin inhibition: does it result in harmful effects by the (pro)renin receptor?" Hypertension research : official journal of the Japanese Society of Hypertension **33**(1): 4-10.

Danser, AH, JP van Kats, PJ Admiraal, FH Derkx, JM Lamers, PD Verdouw, PR Saxena and MA Schalekamp (1994). "Cardiac renin and angiotensins. Uptake from plasma versus in situ synthesis." Hypertension **24**(1): 37-48.

Davì, G, F Gennaro, A Spatola, I Catalano, M Averna, G Montalto, S Amato and A Notarbartolo (1992). "Thrombin-antithrombin III complexes in type II diabetes mellitus." Journal of Diabetes and its Complications **6**(1): 7-11.

de Zeeuw, D, T Akizawa, P Audhya, GL Bakris, M Chin, H Christ-Schmidt, A Goldsberry, M Houser, M Krauth, HJ Lambers Heerspink, JJ McMurray, CJ Meyer, H-H Parving, G Remuzzi, RD Toto, ND Vaziri, C Wanner, J Wittes, D Wrolstad and GM Chertow (2013). "Bardoxolone Methyl in Type 2 Diabetes and Stage 4 Chronic Kidney Disease." New England Journal of Medicine **369**(26): 2492-2503.

Deen, D (2004). "Metabolic syndrome: time for action." American Family Physician **69**(12): 2875-2882.

DeFronzo, RA and E Ferrannini (1991). "Insulin Resistance: A Multifaceted Syndrome Responsible for NIDDM, Obesity, Hypertension, Dyslipidemia, and Atherosclerotic Cardiovascular Disease." Diabetes care **14**(3): 173-194.

Deshpande, SD, S Putta, M Wang, JY Lai, M Bitzer, RG Nelson, LL Lanting, M Kato and R Natarajan (2013). "Transforming growth factor-beta-induced cross talk between p53 and a microRNA in the pathogenesis of diabetic nephropathy." Diabetes **62**(9): 3151-3162.

Desroches, S and B Lamarche (2007). "The evolving definitions and increasing prevalence of the metabolic syndrome." Applied Physiology, Nutrition, and Metabolism **32**(1): 23-32.

Dieterle, F, E Perentes, A Cordier, DR Roth, P Verdes, O Grenet, S Pantano, P Moulin, D Wahl, A Mahl, P End, F Staedtler, F Legay, K Carl, D Laurie, SD Chibout, J Vonderscher and G Maurer (2010). "Urinary clusterin, cystatin C, beta2-microglobulin and total protein as markers to detect drug-induced kidney injury." Nature biotechnology **28**(5): 463-469.

Doak, CM, TMA Wijnhoven, DF Schokker, TLS Visscher and JC Seidell (2012). "Age standardization in mapping adult overweight and obesity trends in the WHO European Region." Obesity Reviews **13**(2): 174-191.

Dooley, R, BJ Harvey and W Thomas (2012). "Non-genomic actions of aldosterone: from receptors and signals to membrane targets." Molecular and Cellular Endocrinology **350**(2): 223-234.

Drozdzik, M, L Domanski, J Wojcicki, A Pudlo and P Machoy (2003). "Effect of unilateral nephrectomy on the pharmacokinetics of atenolol in humans." Journal of Clinical Parmacology **43**(5): 524-528.

Du, XL, D Edelstein, S Dimmeler, Q Ju, C Sui and M Brownlee (2001). "Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site." The Journal of clinical investigation **108**(9): 1341-1348.

Dunning, BE and JE Gerich (2007). "The role of alpha-cell dysregulation in fasting and postprandial hyperglycemia in type 2 diabetes and therapeutic implications." Endocrinology Review **28**(3): 253-283.

Eardley, KS, D Zehnder, M Quinkler, J Lepenies, RL Bates, CO Savage, AJ Howie, D Adu and P Cockwell (2006). "The relationship between albuminuria, MCP-1/CCL2, and interstitial macrophages in chronic kidney disease." Kidney International **69**(7): 1189-1197.

Eguchi, K and I Manabe (2014). "Toll-like receptor, lipotoxicity and chronic inflammation: the pathological link between obesity and cardiometabolic disease." Journal of Atherosclerosis and Thrombosis **21**(7): 629-639.

Engeli, S, J Böhnke, K Gorzelniak, J Janke, P Schling, M Bader, FC Luft and AM Sharma (2005). "Weight loss and the renin-angiotensin-aldosterone system." Hypertension **45**(3): 356-362.

Epstein, M and DA Calhoun (2011). "Aldosterone Blockers (Mineralocorticoid Receptor Antagonism) and Potassium-Sparing Diuretics." The Journal of Clinical Hypertension **13**(9): 644-648.

Fan, JM, YY Ng, PA Hill, DJ Nikolic-Paterson, W Mu, RC Atkins and HY Lan (1999). "Transforming growth factor-beta regulates tubular epithelial-myofibroblast transdifferentiation in vitro." Kidney International **56**(4): 1455-1467.

Fang, ZY, JB Prins and TH Marwick (2004). "Diabetic cardiomyopathy: evidence, mechanisms, and therapeutic implications." Endocrinology Review **25**(4): 543-567.

Fassett, RG, SK Venuthurupalli, GC Gobe, JS Coombes, MA Cooper and WE Hoy (2011). "Biomarkers in chronic kidney disease: a review." Kidney International **80**(8): 806-821.

Félétou, M and PM Vanhoutte (2006). Endothelial dysfunction: a multifaceted disorder (The Wiggers Award Lecture).

Ferdous, A, PK Battiprolu, YG Ni, BA Rothermel and JA Hill (2010). "FoxO, autophagy, and cardiac remodeling."Journal of Cardiovascular Translational Research **3**(4): 355-364.

Fernandez Juarez, G, J Luño, V Barrio, SG de Vinuesa, M Praga, M Goicoechea, V Cachofeiro, J Nieto, F Fernández Vega, A Tato and E Gutierrez (2013). "Effect of Dual Blockade of the Renin-Angiotensin System on the Progression of Type 2 Diabetic Nephropathy: A Randomized Trial." American journal of kidney diseases **61**(2): 211-218.

Fine, L (1986). "The biology of renal hypertrophy." Kidney International **29**(3): 619-634.

Fioretto, P and M Mauer (2007). "Histopathology of diabetic nephropathy." Seminars in Nephrology **27**(2): 195-207.

Fiorini, G, LA Scotti, ML Parmigiani, M Ferrari, P Pezzoli and G Bignotti (1995). "[An echocardiographic study of left ventricular diastolic function in patients with diabetes mellitus type 2]." Giornale Italiano di Cardiologia **25**(1): 17-25.

Flegal, KM, MD Carroll, BK Kit and CL Ogden (2012). "PRevalence of obesity and trends in the distribution of body mass index among us adults, 1999-2010." JAMA **307**(5): 491-497.

Fong, DS, L Aiello, TW Gardner, GL King, G Blankenship, JD Cavallerano, FL Ferris, 3rd and R Klein (2004). "Retinopathy in diabetes." Diabetes care **27**(1): S84-87.

Forclaz, A, M Maillard, J Nussberger, HR Brunner and M Burnier (2003). "Angiotensin II receptor blockade: is there truly a benefit of adding an ACE inhibitor?" Hypertension **41**(1): 31-36.

Ford, ES, WH Giles and WH Dietz (2002). "Prevalence of the metabolic syndrome among US adults: Findings from the Third National Health and Nutrition Examination Survey." Journal of the American Medical Association **287**(3): 356-359.

Fowler, MJ (2011). "Microvascular and Macrovascular Complications of Diabetes." Clinical diabetes **29**(3): 116-122.

Frayn, KN (2001). "Adipose tissue and the insulin resistance syndrome." Proceedings Of The Nutrition Society **60**(3): 375-380.

Fried, LF, N Emanuele, JH Zhang, M Brophy, TA Conner, W Duckworth, DJ Leehey, PA McCullough, T O'Connor, PM Palevsky, RF Reilly, SL Seliger, SR Warren, S Watnick, P Peduzzi and P Guarino (2013). "Combined Angiotensin Inhibition for the Treatment of Diabetic Nephropathy." New England Journal of Medicine **369**(20): 1892-1903.

Fuchs, TC and P Hewitt (2011). "Biomarkers for drug-induced renal damage and nephrotoxicity-an overview for applied toxicology." The AAPS Journal **13**(4): 615-631.

Fujioka, K (2006). "Metabolic syndrome treatment strategies." Pharmacotherapy **26**(12 Pt 2): 222S-226S.

Gabbay, KH (1975). "Hyperglycemia, polyol metabolism, and complications of diabetes mellitus." Annual Review of Medicine **26**: 521-536.

Geraldes, P and GL King (2010). "Activation of Protein Kinase C Isoforms and Its Impact on Diabetic Complications." Circulation research **106**(8): 1319-1331.

Gerstein, HC, JF Mann, Q Yi, B Zinman, SF Dinneen, B Hoogwerf, JP Halle, J Young, A Rashkow, C Joyce, S Nawaz and S Yusuf (2001). "Albuminuria and risk of cardiovascular events, death, and heart failure in diabetic and nondiabetic individuals." JAMA **286**(4): 421-426.

Giacco, F and M Brownlee (2010). "Oxidative Stress and Diabetic Complications." Circulation research **107**(9): 1058-1070.

Go, AS, GM Chertow, D Fan, CE McCulloch and C-y Hsu (2004). "Chronic Kidney Disease and the Risks of Death, Cardiovascular Events, and Hospitalization." New England Journal of Medicine **351**(13): 1296-1305.

Gobal, F, A Deshmukh, S Shah and JL Mehta (2011). "Triad of Metabolic Syndrome, Chronic Kidney Disease, and Coronary Heart Disease With a Focus on Microalbuminuria: Death by Overeating." Journal of the American College of Cardiology **57**(23): 2303-2308.

Goergen, SK, G Rumbold, G Compton and C Harris (2010). "Systematic review of current guidelines, and their evidence base, on risk of lactic acidosis after administration of contrast medium for patients receiving metformin." Radiology **254**(1): 261-269.

Goodpaster, BH, J He, S Watkins and DE Kelley (2001). "Skeletal Muscle Lipid Content and Insulin Resistance: Evidence for a Paradox in Endurance-Trained Athletes." The Journal of Clinical Endocrinology & Metabolism **86**(12): 5755-5761.

Goossens, GH (2008). "The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance." Physiology & Behavior **94**(2): 206-218.

Goossens, GH (2012). "The renin-angiotensin system in the pathophysiology of type 2 diabetes." Obesity Facts **5**(4): 611-624.

Goossens, GH, EE Blaak, WH Saris and MA van Baak (2004). "Angiotensin II-induced effects on adipose and skeletal muscle tissue blood flow and lipolysis in normal-weight and obese subjects." The Journal of Clinical Endocrinology and Metabolism **89**(6): 2690-2696.

Gottdiener, JS, J Bednarz, R Devereux, J Gardin, A Klein, WJ Manning, A Morehead, D Kitzman, J Oh, M Quinones, NB Schiller, JH Stein and NJ Weissman (2004). "American Society of Echocardiography recommendations for use of echocardiography in clinical trials: A report from the american society of echocardiography's guidelines and standards committee and the task force on echocardiography in clinical trials." Journal of the American Society of Echocardiography **17**(10): 1086-1119.

Goumenos, DS, S Tsakas, AM El Nahas, S Alexandri, S Oldroyd, P Kalliakmani and JG Vlachojannis (2002). "Transforming growth factor-beta(1) in the kidney and urine of patients with glomerular disease and proteinuria." Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association **17**(12): 2145-2152.

Gouni-Berthold, I and HK Berthold (2013). "The role of niacin in lipid-lowering treatment: are we aiming too high?" Current Pharmaceutical Design **19**(17): 3094-3106.

Griffin, KA, M Abu-Naser, I Abu-Amarah, M Picken, GA Williamson and AK Bidani (2007). "Dynamic blood pressure load and nephropathy in the ZSF1 (fa/facp) model of type 2 diabetes." American Journal of Physiology - Renal Physiology **293**(5): F1605-F1613.

Grundy, S, J Cleeman, S Daniels, K Donato, R Eckel, B Franklin, D Gordon, R Krauss, P Savage, S Smith, J Spertus and F Costa (2005). "Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement." Circulation **112**: 2735 - 2752.

Grundy, SM, JI Cleeman, CNB Merz, HB Brewer, LT Clark, DB Hunninghake, RC Pasternak, SC Smith, NJ Stone, ftCCotNCE Program, L Endorsed by the National Heart, ACoCF Blood Institute and AH Association (2004). "Implications of Recent Clinical Trials for the National Cholesterol Education Program Adult Treatment Panel III Guidelines." Circulation **110**(2): 227-239.

Grundy, SM, B Hansen, SC Smith, JI Cleeman, RA Kahn and fC Participants (2004). "Clinical Management of Metabolic Syndrome: Report of the American Heart Association/National Heart, Lung, and Blood Institute/American Diabetes Association Conference on Scientific Issues Related to Management." Circulation **109**(4): 551-556.

Haffner, SM, S Lehto, T Ronnemaa, K Pyorala and M Laakso (1998). "Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction." The New England Journal of Medicine **339**(4): 229-234.

Haffner, SM, RA Valdez, HP Hazuda, BD Mitchell, PA Morales and MP Stern (1992). "Prospective Analysis of The Insulin-Resistance Syndrome (Syndrome X)." Diabetes **41**(6): 715-722.

Hall, JE (1997). "Mechanisms of abnormal renal sodium handling in obesity hypertension." American Journal of Hypertension **10**(5 Pt 2): 49S-55S.

Haller, H (1977). "[Epidermiology and associated risk factors of hyperlipoproteinemia]." Zeitschrift fur die Gesamte Innere Medizin **32**(8): 124-128.

Han, WK, V Bailly, R Abichandani, R Thadhani and JV Bonventre (2002). "Kidney Injury Molecule-1 (KIM-1): a novel biomarker for human renal proximal tubule injury." Kidney International **62**(1): 237-244.

Hanefeld, M and W Leonhardt (1981). "Das Metabolische Syndrom." Dt Gesundh Wesen **36**(545-551).

Hanton G, EV, Rocherfort G, Bonnet P, Hyvelin JM (2008). "Echocardiography, a non-invasive method for the assessment of cardiac function and morphology in preclinical drug toxicology and safety pharmacology." Expert Opinion on Drug Metabolism & Toxicology **4**(6): 681-696.

Hargovan, M and A Ferro (2014). "Aldosterone synthase inhibitors in hypertension: current status and future possibilities." JRSM Cardiovascular Disease. 2014 Feb 5;3:2048004014522440. eCollection 2014 Jan.

Harrison-Bernard, LM (2009). "The renal renin-angiotensin system". Advances in Physiology Education **33**(4): 270-274.

Hellemons, ME, M Mazagova, RT Gansevoort, RH Henning, D de Zeeuw, SJ Bakker, HJ Lambers-Heerspink and LE Deelman (2012). "Growth-differentiation factor 15 predicts worsening of albuminuria in patients with type 2 diabetes." Diabetes care **35**(11): 2340-2346.

Heloisa, M, M Shimizu, TM Coimbra, M de Araujo, LF Menezes and AC Seguro (2005). "N-acetylcysteine attenuates the progression of chronic renal failure." Kidney International **68**(5): 2208-2217.

Henriksen, EJ and M Prasannarong (2013). "The role of the renin-angiotensin system in the development of insulin resistance in skeletal muscle." Molecular and Cellular Endocrinology **378**(1-2): 15-22.

Herrero-Morin, JD, S Malaga, N Fernandez, C Rey, MA Dieguez, G Solis, A Concha and A Medina (2007). "Cystatin C and beta2-microglobulin: markers of glomerular filtration in critically ill children." Critical Care **11**(3).

Heyen, JRR, ER Blasi, K Nikula, R Rocha, HA Daust, G Frierdich, JF Van Vleet, P De Ciechi, EG McMahon and AE Rudolph (2002). "Structural, functional, and molecular characterization of the SHHF model of heart failure." American Journal of Physiology - Heart and Circulatory Physiology **283**(5): H1775-H1784.

Himsworth, HP (1936). "Management of Diabetes Mellitus." British Medical Journal **2**(3942): 188-190.

Hink, U, H Li, H Mollnau, M Oelze, E Matheis, M Hartmann, M Skatchkov, F Thaiss, RAK Stahl, A Warnholtz, T Meinertz, K Griendling, DG Harrison, U Forstermann and T Munzel (2001). "Mechanisms Underlying Endothelial Dysfunction in Diabetes Mellitus." Circulation research **88**(2): e14-e22.

Hitzenberger, K (1921). "Über den Blutdruck bei Diabetes Mellitus." Wiener Archiv fur Innere Medizin **2**: 461-466.

Hitzenberger, K and M Richter-Quittner (1921). "Ein Beitrag zum Stoffwechsel bei der vaskulären Hypertonie." Wiener Archiv fur Innere Medizin **2**: 189–216.

Ho, JE, SJ Hwang, KC Wollert, MG Larson, S Cheng, T Kempf, RS Vasan, JL Januzzi, TJ Wang and CS Fox (2013). "Biomarkers of cardiovascular stress and incident chronic kidney disease." Clinical Chemistry **59**(11): 1613-1620.

Hollenberg, NK, ND Fisher and DA Price (1998). "Pathways for angiotensin II generation in intact human tissue: evidence from comparative pharmacological interruption of the renin system." Hypertension **32**(3): 387-392.

Hossain, P, B Kawar and M El Nahas (2007). "Obesity and Diabetes in the Developing World — A Growing Challenge." New England Journal of Medicine **356**(3): 213-215.

Hsiao, FY, WF Huang, YW Wen, PF Chen, KN Kuo and YW Tsai (2009). "Thiazolidinediones and cardiovascular events in patients with type 2 diabetes mellitus: a retrospective cohort study of over 473,000 patients using the National Health Insurance database in Taiwan." Drug Safety **32**(8): 675-690.

Huang, Y, S Wongamorntham, J Kasting, D McQuillan, RT Owens, L Yu, NA Noble and W Border (2006). "Renin increases mesangial cell transforming growth factor-beta1 and matrix proteins through receptor-mediated, angiotensin II-independent mechanisms." Kidney International **69**(1): 105-113.

Hutley, L and JB Prins (2005). "Fat as an endocrine organ: relationship to the metabolic syndrome." The American Journal of the Medical Sciences **330**(6): 280-289.

Ichihara, A and K Kinouchi (2011). "Current knowledge of (pro)renin receptor as an accessory protein of vacuolar H+-ATPase." JRAAS - Journal of the Renin-Angiotensin-Aldosterone System **12**(4): 638-640.

IDF (2005) "The IDF consensus worldwide definition of the metabolic syndrome."

IDF (2014). "The IDF consensus worldwide definition of the metabolic syndrome".

Inoguchi, T, P Li, F Umeda, HY Yu, M Kakimoto, M Imamura, T Aoki, T Etoh, T Hashimoto, M Naruse, H Sano, H Utsumi and H Nawata (2000). "High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C--dependent activation of NAD(P)H oxidase in cultured vascular cells." Diabetes **49**(11): 1939-1945.

investigators, TH (2000). "Effects of ramipril on cardiovascular and microvascular outcomes in people with diabetes mellitus: results of the HOPE study and MICRO-HOPE substudy. Heart Outcomes Prevention Evaluation Study Investigators." Lancet **355**(9200): 253-259.

Israili, ZH, B Lyoussi, R Hernandez-Hernandez and M Velasco (2007). "Metabolic syndrome: treatment of hypertensive patients." American journal of therapeutics **14**(4): 386-402.

Izzo, JL, Jr., MS Weinberg, JW Hainer, J Kerkering and CK Tou (2004). "Antihypertensive efficacy of candesartan-lisinopril in combination vs. up-titration of lisinopril: the AMAZE trials." The Journal of Clinical Hypertension **6**(9): 485-493.

James, PA, S Oparil, BL Carter and et al. (2014). "2014 evidence-based guideline for the management of high blood pressure in adults: Report from the panel members appointed to the eighth joint national committee (jnc 8)." JAMA **311**(5): 507-520.

Janghorbani, M, FB Hu, WC Willett, TY Li, JE Manson, G Logroscino and KM Rexrode (2007). "Prospective Study of Type 1 and Type 2 Diabetes and Risk of Stroke Subtypes: The Nurses' Health Study." Diabetes care **30**(7): 1730-1735.

Jaramillo, MC and DD Zhang (2013). "The emerging role of the Nrf2–Keap1 signaling pathway in cancer." Genes & Development **27**(20): 2179-2191.

Jensen, C, P Herold and HR Brunner (2008). "Aliskiren: the first renin inhibitor for clinical treatment." Nature Reviews Drug Discovery **7**(5): 399-410.

Jha, V, G Garcia-Garcia, K Iseki, Z Li, S Naicker, B Plattner, R Saran, AY-M Wang and C-W Yang (2013). "Chronic kidney disease: global dimension and perspectives." The Lancet **382**(9888): 260-272.

Joshi, D, R Gupta, A Dubey, A Shiwalkar, P Pathak, RC Gupta, V Chauthaiwale and C Dutt (2009). "TRC4186, a novel AGE-breaker, improves diabetic cardiomyopathy and nephropathy in Ob-ZSF1 model of type 2 diabetes." Journal of Cardiovascular Pharmacology **54**(1): 72-81.

Kaplan, NM (1989). "The deadly quartet. Upper body obesity, glucose intolerance, hypertriglyceridemia and hypertension." Archives of Internal Medicine **149**: 1514–1520.

Karlsson, C, K Lindell, M Ottosson, L Sjostrom, B Carlsson and LM Carlsson (1998). "Human adipose tissue expresses angiotensinogen and enzymes required for its conversion to angiotensin II." The Journal of Clinical Endocrinology and Metabolism **83**(11): 3925-3929.

Karns, AD, JM Bral, D Hartman, T Peppard and C Schumacher (2013). "Study of aldosterone synthase inhibition as an add-on therapy in resistant hypertension." The Journal of Clinical Hypertension **15**(3): 186-192.

Kashyap, ML, ME McGovern, K Berra, JR Guyton, PO Kwiterovich Jr, WL Harper, PD Toth, LK Favrot, B Kerzner, SD Nash, HE Bays and PD Simmons (2002). "Long-term safety and efficacy of a once-daily niacin/lovastatin formulation for patients with dyslipidemia*." The American journal of cardiology **89**(6): 672-678.

Kasiske, BL, MP O'Donnell, MP Cleary, WF Keane and F Phillips (1989). "Effects of reduced renal mass on tissue lipids and renal injury in hyperlipidemic rats." Kidney International **35**(1): 40-47.

Kasuga, M (2006). "Insulin resistance and pancreatic beta cell failure." Journal of Clinical Investigation **116**(7): 1756-1760.

Katagiri, H, T Yamada and Y Oka (2007). "Adiposity and cardiovascular disorders: disturbance of the regulatory system consisting of humoral and neuronal signals." Circulation Research **101**(1): 27-39.

Katsuda, Y, Y Kemmochi, M Maki, R Sano, Y Toriniwa, Y Ishii, K Miyajima, K Kakimoto and T Ohta (2014). "Effects of Unilateral Nephrectomy on Renal Function in Male Spontaneously Diabetic Torii Fatty Rats: A Novel Obese Type 2 Diabetic Model." Journal of Diabetes Research **2014**: 6.

Kaur, J (2014). "A Comprehensive Review on Metabolic Syndrome." Cardiology Research and Practice **2014**: 21.

KDOQI (2002). "K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification." American journal of kidney diseases : the official journal of the National Kidney Foundation **39**(2 Suppl 1): S1-266.

Keech, A, RJ Simes, P Barter, J Best, R Scott, MR Taskinen, P Forder, A Pillai, T Davis, P Glasziou, P Drury, YA Kesaniemi, D Sullivan, D Hunt, P Colman, M d'Emden, M Whiting, C Ehnholm and M Laakso (2005). "Effects of long-term fenofibrate therapy on cardiovascular events in 9795 people with type 2 diabetes mellitus (the FIELD study): randomised controlled trial." Lancet **366**(9500): 1849-1861.

Kempf, T and KC Wollert (2009). "Growth-differentiation factor-15 in heart failure." Heart Failure Clinics **5**(4): 537-547.

Kershaw, EE and JS Flier (2004). "Adipose Tissue as an Endocrine Organ." The Journal of Clinical Endocrinology & Metabolism **89**(6): 2548-2556.

Kilhovd, BK, TJ Berg, KI Birkeland, P Thorsby and KF Hanssen (1999). "Serum levels of advanced glycation end products are increased in patients with type 2 diabetes and coronary heart disease." Diabetes care **22**(9): 1543-1548.

Kirk, R (2013). "Renal fibrosis: KIM-1 expression links kidney injury with CKD in mice." Nature reviews. Nephrology **9**(11): 627.

Kobori, H, M Nangaku, LG Navar and A Nishiyama (2007). "The Intrarenal Renin-Angiotensin System: From Physiology to the Pathobiology of Hypertension and Kidney Disease." Pharmacological Reviews **59**(3): 251-287.

Kobori, H, Y Ozawa, Y Suzaki, MC Prieto-Carrasquero, A Nishiyama, T Shoji, EP Cohen and LG Navar (2006). Young Scholars Award Lecture: Intratubular angiotensinogen in hypertension and kidney diseases, Am Journal of Hypertension. 2006 May;19(5):541-50.

Kolovou, GD, KK Anagnostopoulou, KD Salpea and DP Mikhailidis (2007). "The Prevalence of Metabolic Syndrome in Various Populations." The American Journal of the Medical Sciences **333**(6): 362-371 310.1097/MAJ.1090b1013e318065c318063a318061.

Koplan, JP and WH Dietz (1999). "CAloric imbalance and public health policy." JAMA **282**(16): 1579-1581.

Kouroedov, A, M Eto, H Joch, M Volpe, TF Lüscher and F Cosentino (2004). "Selective Inhibition of Protein Kinase Cβ2 Prevents Acute Effects of High Glucose on Vascular Cell Adhesion Molecule-1 Expression in Human Endothelial Cells." Circulation **110**(1): 91-96.

Krogh-Madsen, R, P Plomgaard, K Moller, B Mittendorfer and BK Pedersen (2006). "Influence of TNF-alpha and IL-6 infusions on insulin sensitivity and expression of IL-18 in humans." American Journal of Physiology: Endocrinology and Metabolism **291**(1): 7.

Kylin, E (1921). "Hypertonie and Zuckerkrankheit." Zentralblatt für Innere Medizin **42**: 873–877.

Kylin, E (1923). "Studien über das Hypertoni-Hyperglycemi-Hyperurikemi syndrom." Zentralblatt für Innere Medizin **44**: 105-112.

Laakso, M (1999). "Hyperglycemia and cardiovascular disease in type 2 diabetes." Diabetes **48**(5): 937-942.

Lajer, M, A Jorsal, L Tarnow, H-H Parving and P Rossing (2010). "Plasma Growth Differentiation Factor-15 Independently Predicts All-Cause and Cardiovascular Mortality As Well As Deterioration of Kidney Function in Type 1 Diabetic Patients With Nephropathy." Diabetes care **33**(7): 1567-1572.

Lamarche, B, S Rashid and GF Lewis (1999). "HDL metabolism in hypertriglyceridemic states: an overview." Clinica Chimica Acta **286**(1-2): 145-161.

Lambers Heerspink, HJ, GM Chertow, T Akizawa, P Audhya, GL Bakris, A Goldsberry, M Krauth, P Linde, JJ McMurray, CJ Meyer, HH Parving, G Remuzzi, H Christ-Schmidt, RD Toto, ND Vaziri, C Wanner, J Wittes, D Wrolstad and D de Zeeuw (2013). "Baseline characteristics in the Bardoxolone methyl EvAluation in patients with Chronic kidney disease and type 2 diabetes mellitus: the Occurrence of renal eveNts (BEACON) trial." Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association **28**(11): 2841-2850.

Lastra, G, S Dhuper, MS Johnson and JR Sowers (2010). "Salt, aldosterone, and insulin resistance: impact on the cardiovascular system." Nature Reviews Cardiology **7**(10): 577-584.

Lau, DC, B Dhillon, H Yan, PE Szmitko and S Verma (2005). "Adipokines: molecular links between obesity and atheroslcerosis." AJP Heart and Circulatory Physiology **288**(5): 14.

Lavoz, C, R Rodrigues-Diez, A Benito-Martin, S Rayego-Mateos, RR Rodrigues-Diez, M Alique, A Ortiz, S Mezzano, J Egido and M Ruiz-Ortega (2012). "Angiotensin II contributes to renal fibrosis independently of Notch pathway activation." PLoS ONE **7**(7): 9.

Le Goff, W, M Guerin and MJ Chapman (2004). "Pharmacological modulation of cholesteryl ester transfer protein, a new therapeutic target in atherogenic dyslipidemia." Nature Reviews Cardiology **101**(1): 17-38.

Lee, SB, K Kanasaki and R Kalluri (2009). "Circulating TGF-beta1 as a reliable biomarker for chronic kidney disease progression in the African-American population." Kidney International **76**(1): 10-12.

Lemarié, CA and EL Schiffrin (2010). "The angiotensin II type 2 receptor in cardiovascular disease." Journal of Renin-Angiotensin-Aldosterone System **11**(1): 19-31.

Leung, PS (2007). "Mechanisms of protective effects induced by blockade of the renin-angiotensin system: novel role of the pancreatic islet angiotensin-generating system in Type 2 diabetes." Diabetic medicine : a journal of the British Diabetic Association **24**(2): 110-116.

Lewis, GF and G Steiner (1996). "Acute effects of insulin in the control of VLDL production in humans. Implications for the insulin-resistant state." Diabetes care **19**(4): 390-393.

Liebson, PR and EA Amsterdam (2009). "Ongoing Telmisartan Alone and in Combination With Ramipril Global Endpoint Trial (ONTARGET): implications for reduced cardiovascular risk." Preventive Cardiology **12**(1): 43-50.

Lien, LF, AJ Brown, JD Ard, C Loria, TP Erlinger, AC Feldstein, P-H Lin, CM Champagne, AC King, HL McGuire, VJ Stevens, PJ Brantley, DW Harsha, MA McBurnie, LJ Appel and LP Svetkey (2007). "Effects of PREMIER Lifestyle Modifications on Participants With and Without the Metabolic Syndrome." Hypertension **50**(4): 609-616.

Lindholm, LH, M Persson, P Alaupovic, B Carlberg, A Svensson and O Samuelsson (2003). "Metabolic outcome during 1 year in newly detected hypertensives: results of the Antihypertensive Treatment and Lipid Profile in a North of Sweden Efficacy Evaluation (ALPINE study)." Journal of Hypertension **21**(8): 1563-1574.

Liu, M and F Liu (2009). "Transcriptional and post-translational regulation of adiponectin." Biochemical Journal **425**(1): 41-52.

Lock, EA (2010). "Sensitive and early markers of renal injury: where are we and what is the way forward?" Toxicological Sciences **116**(1): 1-4.

Loeffler, I and G Wolf (2014). "Transforming growth factor-β and the progression of renal disease." Nephrology Dialysis Transplantation **29**(suppl 1): i37-i45.

Lopes, GS, CC Lemos, CA Mandarim-De-Lacerda and R Bregman (2004). "Effect of unilateral nephrectomy on renal function of diabetic rats." Histology and Histopathology **19**(4): 1085-1088.

López, Y, MJ Paloma, J Rifón, B Cuesta and JA Páramo (1999). "Measurement of Prethrombotic Markers in the Assessment of Acquired Hypercoagulable States." Thrombosis Research **93**(2): 71-78.

Luk, CC-W, K-M Chow, JS-S Kwok, BC-H Kwan, MH-M Chan, K-B Lai, FM-M Lai, G Wang, PK-T Li and C-C Szeto (2013). "Urinary Biomarkers for The Prediction of Reversibility in Acute-on-Chronic Renal Failure." Disease Markers **34**(3).

Mahmud, A and J Feely (2004). "Arterial stiffness and the renin-angiotensin-aldosterone system." Journal of the Renin-Angiotensin-Aldosterone System **5**(3):102-8.

Maillard, D (1912). "Action des acides aminés sur les sucres: formation des melanoidines par voie methodique." Comptes rendus hebdomadaires des séances de l'Académie des sciences **154**: 66-68.

Malhotra, A, BP Kang, S Cheung, D Opawumi and LG Meggs (2001). "Angiotensin II promotes glucose-induced activation of cardiac protein kinase C isozymes and phosphorylation of troponin I." Diabetes **50**(8): 1918-1926.

Malmberg, K, S Yusuf, HC Gerstein, J Brown, F Zhao, D Hunt, L Piegas, J Calvin, M Keltai and A Budaj (2000). "Impact of diabetes on long-term prognosis in patients with unstable angina and non-Q-wave myocardial infarction: results of the OASIS (Organization to Assess Strategies for Ischemic Syndromes) Registry." Circulation **102**(9): 1014-1019.

Manrique, C, G Lastra, M Gardner and JR Sowers (2009). "The renin angiotensin aldosterone system in hypertension: roles of insulin resistance and oxidative stress." Medical Clinics of North America **93**(3): 569-582.

Marañon, G (1922). "Über Hypertonie and Zuckerkrankheit." Zentralblatt für Innere Medizin **43**: 169–176.

Marsh, AJ, MAP Fontes, S Killinger, DB Pawlak, JW Polson and RAL Dampney (2003). "Cardiovascular responses evoked by leptin acting on neurons in the ventromedial and dorsomedial hypothalamus." Hypertension **42**(4 I): 488-493.

Marsh, SA, PC Powell, A Agarwal, LJ Dell'Italia and JC Chatham (2007). "Cardiovascular dysfunction in Zucker obese and Zucker diabetic fatty rats: role of hydronephrosis." American Journal of Physiology - Heart and Circulatory Physiology **293**(1): H292-H298.

Matsushita, K, M van der Velde, BC Astor, M Woodward, AS Levey, PE de Jong, J Coresh and RT Gansevoort (2010). "Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis." Lancet **375**(9731): 2073-2081.

Matsuzawa, Y (2005). "Metabolic syndrome--definition and diagnostic criteria in Japan." Journal of Atherosclerosis and Thrombosis **12**(6): 301.

McGill, JB, DJ Schneider, CL Arfken, CL Lucore and BE Sobel (1994). "Factors Responsible for Impaired Fibrinolysis in Obese Subjects and NIDDM Patients." Diabetes **43**(1): 104-109.

Mehnert, H and H Kuhlmann (1968). "[Hypertension and diabetes mellitus]." Deutsche Medizinische Journal **19**(16): 567-571.

Menard, J (2004). "The 45-year story of the development of an anti-aldosterone more specific than spironolactone." Molecular and Cellular Endocrinology **217**(1-2): 45-52.

Merritt, C, Ed. (1998). Physics of ultrasound. Diagnostic ultrasound. St.Louis.

Metcalfe, W (2007). "How does early chronic kidney disease progress?: A Background Paper prepared for the UK Consensus Conference on Early Chronic Kidney Disease." Nephrology Dialysis Transplantation **22**(suppl 9): ix26-ix30.

Milan, A, F Tosello, A Fabbri, A Vairo, D Leone, M Chiarlo, M Covella and F Veglio (2011). "Arterial stiffness: from physiology to clinical implications." High Blood Pressure & Cardiovascular Prevention **18**(1): 1-12.

Moncada, S and EA Higgs (2006). Nitric Oxide and the Vascular Endothelium. The Vascular Endothelium I. S. Moncada and A. Higgs, Springer Berlin Heidelberg. **176/I:** 213-254.

Montezano, AC and RM Touyz (2008). "Networking between systemic angiotensin II and cardiac mineralocorticoid receptors."Hypertension **52**(6):1016-1018.

Morgan, L, F Broughton Pipkin and N Kalsheker (1996). "Angiotensinogen: molecular biology, biochemistry and physiology." The International Journal of Biochemistry & Cell Biology **28**(11): 1211-1222.

Mufunda, J, R Chatora, Y Ndambakuwa, P Nyarango, A Kosia, J Chifamba, A Filipe, A Usman and VH Sparks (2006). "Emerging non-communicable disease epidemic in Africa: preventive measures from the WHO Regional Office for Africa." Ethnicity & Disease **16**(2): 521-526.

Mulder, P, B Devaux, L El Fertak, P Compagnon, V Richard, J-P Henry, E Scalbert, P Desché, B Macé and C Thuillez (1995). "Vascular and myocardial protective effects of converting

enzyme inhibition in experimental heart failure." American Journal of Cardiology **76**(15): 28E-33E.

Mulder, P, V Mellin, J Favre, M Vercauteren, I Remy-Jouet, C Monteil, V Richard, S Renet, JP Henry, AY Jeng, RL Webb and C Thuillez (2008). "Aldosterone synthase inhibition improves cardiovascular function and structure in rats with heart failure: a comparison with spironolactone." European Heart Journal **29**(17): 2171-2179.

Muniyappa, R and S Yavuz (2013). "Metabolic actions of angiotensin II and insulin: a microvascular endothelial balancing act." Molecular and Cellular Endocrinology **378**(1-2): 59-69.

Nagai, T (1994). "Change of lipoprotein (a) and coagulative or fibrinolytic parameters in diabetic patients with nephropathy." Journal of Atherosclerosis and Thrombosis **1**(1): 37-40.

Naka, Y, LG Bucciarelli, T Wendt, LK Lee, LL Rong, R Ramasamy, SF Yan and AM Schmidt (2004). "RAGE axis: Animal models and novel insights into the vascular complications of diabetes." Arteriosclerosis, Thrombosis, and Vascular Biology **24**(8): 1342-1349.

Namsolleck, P and T Unger (2014). "Aldosterone synthase inhibitors in cardiovascular and renal diseases." Nephrology Dialysis Transplantation **29**(suppl 1): i62-i68.

Naresh, VV, AL Reddy, G Sivaramakrishna, PV Sharma, RV Vardhan and VS Kumar (2009). "Angiotensin converting enzyme gene polymorphism in type II diabetics with nephropathy." Indian Journal of Nephrology **19**(4): 145-148.

Nathan, DM, JB Buse, MB Davidson, RJ Heine, RR Holman, R Sherwin and B Zinman (2006). "Management of Hyperglycemia in Type 2 Diabetes: A Consensus Algorithm for the Initiation and Adjustment of Therapy: A consensus statement from the American Diabetes Association and the European Association for the Study of Diabetes." Diabetes care **29**(8): 1963-1972.

Ng, M, T Fleming, M Robinson, B Thomson, N Graetz, C Margono, EC Mullany, S Biryukov, C Abbafati, SF Abera, JP Abraham, NME Abu-Rmeileh, T Achoki, FS AlBuhairan, ZA Alemu, R Alfonso, MK Ali, R Ali, NA Guzman, W Ammar, P Anwari, A Banerjee, S Barquera, S Basu, DA Bennett, Z Bhutta, J Blore, N Cabral, IC Nonato, J-C Chang, R Chowdhury, KJ Courville, MH Criqui, DK Cundiff, KC Dabhadkar, L Dandona, A Davis, A Dayama, SD Dharmaratne, EL Ding, AM Durrani, A Esteghamati, F Farzadfar, DFJ Fay, VL Feigin, A Flaxman, MH Forouzanfar, A Goto, MA Green, R Gupta, N Hafezi-Nejad, GJ Hankey, HC Harewood, R Havmoeller, S Hay, L Hernandez, A Husseini, BT Idrisov, N Ikeda, F Islami, E Jahangir, SK Jassal, SH Jee, M Jeffreys, JB Jonas, EK Kabagambe, SEAH Khalifa, AP Kengne, YS Khader, Y-H Khang, D Kim, RW Kimokoti, JM Kinge, Y Kokubo, S Kosen, G Kwan, T Lai, M Leinsalu, Y Li, X Liang, S Liu, G Logroscino, PA Lotufo, Y Lu, J Ma, NK Mainoo, GA Mensah, TR Merriman, AH Mokdad, J Moschandreas, M Naghavi, A Naheed, D Nand, KMV Narayan, EL Nelson, ML Neuhouser, MI Nisar, T Ohkubo, SO Oti, A Pedroza, D Prabhakaran, N Roy, U Sampson, H Seo, SG Sepanlou, K Shibuya, R Shiri, I Shiue, GM Singh, JA Singh, V

Skirbekk, NJC Stapelberg, L Sturua, BL Sykes, M Tobias, BX Tran, L Trasande, H Toyoshima, S van de Vijver, TJ Vasankari, JL Veerman, G Velasquez-Melendez, VV Vlassov, SE Vollset, T Vos, C Wang, X Wang, E Weiderpass, A Werdecker, JL Wright, YC Yang, H Yatsuya, J Yoon, S-J Yoon, Y Zhao, M Zhou, S Zhu, AD Lopez, CJL Murray and E Gakidou (2014). "Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: a systematic analysis for the Global Burden of Disease Study 2013." The Lancet **384**(9945): 766-781.

Nguyen, DUY, FU Ping, WEI Mu, P Hill, RC Atkins and SJ Chadban (2006). "Macrophage accumulation in human progressive diabetic nephropathy." Nephrology **11**(3): 226-231.

Nguyen, G, F Delarue, C Burckle, L Bouzhir, T Giller and JD Sraer (2002). "Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin." Journal of Clinical Investigation **109**(11): 1417-1427.

Ni, YG, N Wang, DJ Cao, N Sachan, DJ Morris, RD Gerard, OM Kuro, BA Rothermel and JA Hill (2007). "FoxO transcription factors activate Akt and attenuate insulin signaling in heart by inhibiting protein phosphatases." Proceedings of the National Academy of Sciences of the USA **104**(51): 20517-20522.

NIH (1998). "Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults--The Evidence Report. National Institutes of Health." Obesity Research **6**(2): 51S-209S.

Ninichuk, V, O Kulkarni, S Clauss and H Anders (2007). "Tubular atrophy, interstitial fibrosis, and inflammation in type 2 diabetic db/db mice. An accelerated model of advanced diabetic nephropathy." European journal of medical research **12**(8): 351.

Nishikawa, T, D Edelstein, XL Du, S Yamagishi, T Matsumura, Y Kaneda, MA Yorek, D Beebe, PJ Oates, HP Hammes, I Giardino and M Brownlee (2000). "Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage." Nature **404**(6779): 787-790.

Niwa, T, T Katsuzaki, S Miyazaki, T Miyazaki, Y Ishizaki, F Hayase, N Tatemichi and Y Takei (1997). "Immunohistochemical detection of imidazolone, a novel advanced glycation end product, in kidneys and aortas of diabetic patients." The Journal of clinical investigation **99**(6): 1272-1280.

O'Donnell, MP, BL Kasiske, FX Daniels and WF Keane (1986). "Effects of Nephron Loss on Glomerular Hemodynamics and Morphology in Diabetic Rats." Diabetes **35**(9): 1011-1015.

O'Donnell, MP, BL Kasiske and WF Keane (1988). "Glomerular hemodynamic and structural alterations in experimental diabetes mellitus." FASEB Journal **2**(8): 2339-2347.

Ogihara, T, T Asano, K Ando, Y Chiba, H Sakoda, M Anai, N Shojima, H Ono, Y Onishi, M Fujishiro, H Katagiri, Y Fukushima, M Kikuchi, N Noguchi, H Aburatani, I Komuro and T Fujita (2002). "Angiotensin II-induced insulin resistance is associated with enhanced insulin signaling." Hypertension **40**(6): 872-879.

Opie, LH and R Schall (2004). "Old antihypertensives and new diabetes." Journal of Hypertension **22**(8): 1453-1458.

Orsic, V, M Mihalj, M Mogus, H Mihalj, M Jakic, M Heffer-Lauc, K Marjanovic and L Zibar (2011). "Impaired kidney function in rats six months after unilateral nephrectomy - an old story, a new perspective." Medicinski glasnik : official publication of the Medical Association of Zenica-Doboj Canton, Bosnia and Herzegovina **8**(2): 185-191.

Ortiz-Melo, DI and RF Spurney (2014). "Special deLIVERy: podocyte injury promotes renal angiotensin II generation from liver-derived angiotensinogen." Kidney International **85**(5): 1009-1011.

Pagliaro, P and C Penna (2005). "Rethinking the renin-angiotensin system and its role in cardiovascular regulation." Cardiovascular Drugs and Therapy **19**(1): 77-87.

Palaniappan, L, MR Carnethon, Y Wang, AJG Hanley, SP Fortmann, SM Haffner and L Wagenknecht (2004). "Predictors of the Incident Metabolic Syndrome in Adults: The Insulin Resistance Atherosclerosis Study." Diabetes care **27**(3): 788-793.

Paletas, K, E Athanasiadou, M Sarigianni, P Paschos, A Kalogirou, M Hassapidou and A Tsapas (2010). "The protective role of the mediterranean diet on the prevalence of metabolic syndrome in a population of greek obese subjects." Journal of the American College of Nutrition **29**(1): 41-45.

Palm, F, P Liss, A Fasching, P Hansell and PO Carlsson (2001). "Transient glomerular hyperfiltration in the streptozotocin-diabetic Wistar Furth rat." Upsala Journal of Medical Sciences **106**(3): 175-182.

Paneni, F, JA Beckman, MA Creager and F Cosentino (2013). "Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part I." European Heart Journal **34**(31): 2436-2443.

Paneni, F, P Mocharla, A Akhmedov, S Costantino, E Osto, M Volpe, TF Lüscher and F Cosentino (2012). "Gene Silencing of the Mitochondrial Adaptor p66Shc Suppresses Vascular Hyperglycemic Memory in Diabetes." Circulation research **111**(3): 278-289.

Parikh, RM and V Mohan (2012). "Changing definitions of metabolic syndrome." Indian Journal of Endocrinology and Metababolism **16**(1): 7-12.

Park, YW, S Zhu, L Palaniappan, S Heshka, MR Carnethon and SB Heymsfield (2003). "The metabolic syndrome: prevalence and associated risk factor findings in the US population from the Third National Health and Nutrition Examination Survey, 1988-1994." Archives of Internal Medicine **163**(4): 427-436.

Parving, HH, JB Lewis, M Ravid, G Remuzzi and LG Hunsicker (2006). "Prevalence and risk factors for microalbuminuria in a referred cohort of type II diabetic patients: a global perspective." Kidney International **69**(11): 2057-2063.

Paul, M, A Poyan Mehr and R Kreutz (2006). "Physiology of local renin-angiotensin systems." Physiological Reviews **86**(3): 747-803.

Pearlman, AS, T Ryan, MH Picard and PS Douglas (2007). "Evolving trends in the use of echocardiography: a study of Medicare beneficiaries." Journal of the American College of Cardiology **49**(23): 2283-2291.

Pergola, PE, M Krauth, JW Huff, DA Ferguson, S Ruiz, CJ Meyer and DG Warnock (2011). "Effect of bardoxolone methyl on kidney function in patients with T2D and Stage 3b-4 CKD." American journal of nephrology **33**(5): 469-476.

Pergola, PE, P Raskin, RD Toto, CJ Meyer, JW Huff, EB Grossman, M Krauth, S Ruiz, P Audhya, H Christ-Schmidt, J Wittes and DG Warnock (2011). "Bardoxolone methyl and kidney function in CKD with type 2 diabetes." The New England journal of medicine **365**(4): 327-336.

Perico, N, A Benigni and G Remuzzi (2008). "Present and future drug treatments for chronic kidney diseases: evolving targets in renoprotection." Nature Reviews Drug Discovery **7**(11): 936-953.

Persson, F, P Rossing, KJ Schjoedt, T Juhl, L Tarnow, CD Stehouwer, C Schalkwijk, F Boomsma, E Frandsen and HH Parving (2008). "Time course of the antiproteinuric and antihypertensive effects of direct renin inhibition in type 2 diabetes." Kidney International **73**(12): 1419-1425.

Pischon, T, CJ Girman, GS Hotamisligil, N Rifai, FB Hu and EB Rimm (2004). "Plasma adiponectin levels and risk of myocardial infarction in men." JAMA **291**(14): 1730-1737.

Pitt, B, W Remme, F Zannad, J Neaton, F Martinez, B Roniker, R Bittman, S Hurley, J Kleiman and M Gatlin (2003). "Eplerenone, a Selective Aldosterone Blocker, in Patients with Left Ventricular Dysfunction after Myocardial Infarction." New England Journal of Medicine **348**(14): 1309-1321.

Pitt, B, F Zannad, WJ Remme, R Cody, A Castaigne, A Perez, J Palensky and J Wittes (1999). "The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators."The New England Journal of Medicine **341**(10): 709-717.

Pollock, JS and PK Carmines (2008). "Diabetic nephropathy: nitric oxide and renal medullary hypoxia." American Journal of Physiology - Renal Physiology **294**(1): F28-F29.

Pories, WJ, JH Mehaffey and KM Staton (2011). "The surgical treatment of type two diabetes mellitus." Surgical Clinics of North America **91**(4): 821-836.

Prabhakar, S, J Starnes, S Shi, B Lonis and R Tran (2007). "Diabetic nephropathy is associated with oxidative stress and decreased renal nitric oxide production." Journal of the American Society of Nephrology : JASN **18**(11): 2945-2952.

Preiss, D, N Sattar and JJ McMurray (2011). "A systematic review of event rates in clinical trials in diabetes mellitus: The importance of quantifying baseline cardiovascular disease history and proteinuria and implications for clinical trial design." American heart journal **161**(1): 210-219.e211.

Prentki, M and CJ Nolan (2006). "Islet beta cell failure in type 2 diabetes." Journal of Clinical Investigation **116**(7): 1802-1812.

Rafiq, K, H Hitomi, D Nakano, A Ichihara and A Nishiyama (2011). "Possible involvement of the (pro)renin receptor-dependent system in the development of insulin resistance." Frontiers in Bioscience - Scholar **3 S**(4): 1478-1485.

Rafiq, K, H Mori, T Masaki and A Nishiyama (2013). "(Pro)renin receptor and insulin resistance: Possible roles of angiotensin II-dependent and -independent pathways." Molecular and Cellular Endocrinology **378**(1–2): 41-45.

Raimundo, M and JA Lopes (2011). "Metabolic Syndrome, Chronic Kidney Disease, and Cardiovascular Disease: A Dynamic and Life-Threatening Triad." Cardiology Research and Practice **2011**.

Ran, J, T Hirano, T Fukui, K Saito, H Kageyama, K Okada and M Adachi (2006). "Angiotensin II infusion decreases plasma adiponectin level via its type 1 receptor in rats: an implication for hypertension-related insulin resistance." Metabolism: clinical and experimental **55**(4): 478-488.

Randle, PJ, PB Garland, CN Hales and EA Neswholme (1963). "The glucose–fatty acid cycle: its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus." Lancet **1**: 785–789.

Rask-Madsen, C and GL King (2010). "Kidney complications: factors that protect the diabetic vasculature." Nature Medicine **16**(1): 40-41.

Reaven, GM (1988). "Banting lecture 1988 Role of insulin resistance in human disease." Diabetes **37**(12): 1595-1607.

Remuzzi, G, A Benigni and A Remuzzi (2006). "Mechanisms of progression and regression of renal lesions of chronic nephropathies and diabetes." The Journal of clinical investigation **116**(2): 288-296.

Retnakaran, R, CA Cull, KI Thorne, AI Adler and RR Holman (2006). "Risk factors for renal dysfunction in type 2 diabetes: U.K. Prospective Diabetes Study 74." Diabetes **55**(6): 1832-1839.

Reudelhuber, TL (2005). "The renin-angiotensin system: peptides and enzymes beyond angiotensin II." Current opinion in nephrology and hypertension **14**(2): 155-159.

Reutens, AT and RC Atkins (2011). "Epidemiology of diabetic nephropathy." Contrib Nephrol **170**: 1-7.

Riboulet, W, E Hainaut, P Verry, J Lambotte, B Jacobsen, I Lartaud, A Bénardeau and K Conde-Knape (2013). "Longitudinal assessment of heart function of ZSF1 rats by echocardiography." Journal of Experimental and Applied Animal Science **1**(1): 125-139.

Ritz, E and A Tomaschitz (2013). "Aldosterone and kidney: a rapidly moving frontier (an update)." Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association **4**: 4.

Roscioni, SS, HJL Heerspink and D de Zeeuw (2014). "The effect of RAAS blockade on the progression of diabetic nephropathy." Nature reviews. Nephrology **10**(2): 77-87.

Rossing, P (2006). "Diabetic Nephropathy: Worldwide epidemic and effects of current treatment on natural history." Current Diabetes Reports **6**(6): 479-483.

Rossing, P (2013). "Diabetic nephropathy: Could problems with bardoxolone methyl have been predicted?" Nature reviews. Nephrology **9**: 128–130.

Rugale, C, M Cordaillat, A Mimran and B Jover (2005). "Prevention and reversal by enalapril of target organ damage in angiotensin II hypertension." Journal of the renin-angiotensin-aldosterone system : JRAAS **6**(3): 154-160.

Ruilope, LM, J Segura and EL Schiffrin (2003). "ACE inhibition or angiotensin receptor blockade: which should we use in diabetic patients?" Journal of the renin-angiotensin-aldosterone system : JRAAS **4**(2): 74-79.

Russo, C, Z Jin, S Homma, T Rundek, MSV Elkind, RL Sacco and MR Di Tullio (2010). "Effect of diabetes and hypertension on left ventricular diastolic function in a high-risk population without evidence of heart disease." European journal of heart failure **12**(5): 454-461.

Rüster, C and G Wolf (2006). "Renin-Angiotensin-Aldosterone System and Progression of Renal Disease." Journal of the American Society of Nephrology **17**(11): 2985-2991.

Rutter, MK and RW Nesto (2011). "Blood pressure, lipids and glucose in type 2 diabetes: how low should we go? Re-discovering personalized care." European Heart Journal **32**(18): 2247-2255.

Samuel, Varman T and Gerald I Shulman (2012). "Mechanisms for Insulin Resistance: Common Threads and Missing Links." Cell **148**(5): 852-871.

Santoro, D, A Natali, C Palombo, LS Brandi, M Piatti, S Ghione and E Ferrannini (1992). "Effects of chronic angiotensin converting enzyme inhibition on glucose tolerance and insulin sensitivity in essential hypertension." Hypertension **20**(2): 181-191.

Sarafidis, PA and GL Bakris (2007). "Review: Insulin and endothelin: an interplay contributing to hypertension development?" The Journal of Clinical Endocrinology and Metabolism **92**(2): 379-385.

Sarafidis, PA and PM Nilsson (2006). "The metabolic syndrome: a glance at its history." Journal of Hypertension **24**(4): 621-626.

Schena, FP and L Gesualdo (2005). "Pathogenetic Mechanisms of Diabetic Nephropathy." Journal of the American Society of Nephrology **16**(3 suppl 1): S30-S33.

Scheppach, W, C Fabian, F Ahrens, M Spengler and H Kasper (1988). "Effect of starch malabsorption on colonic function and metabolism in humans." Gastroenterology **95**(6): 1549-1555.

Scherrer-Crosbie, M and HB Thibault (2008). "Echocardiography in Translational Research: Of Mice and Men." Journal of the American Society of Echocardiography **21**(10): 1083-1092.

Schillaci, G, M Pirro, G Vaudo, F Gemelli, S Marchesi, C Porcellati and E Mannarino (2004). "Prognostic value of the metabolic syndrome in essential hypertension." Journal of the American College of Cardiology **43**(10): 1817-1822.

Schmidt, AM, SD Yan, J-L Wautier and D Stern (1999). "Activation of Receptor for Advanced Glycation End Products: A Mechanism for Chronic Vascular Dysfunction in Diabetic Vasculopathy and Atherosclerosis." Circulation research **84**(5): 489-497.

Schmieder, RE, KF Hilgers, MP Schlaich and BMW Schmidt (2007). "Renin-angiotensin system and cardiovascular risk." The Lancet **369**(9568): 1208-1219.

Schwartz, MW, SC Woods, D Porte, RJ Seeley and DG Baskin (2000). "Central nervous system control of food intake." Nature **404**(6778): 661-671.

Sechi, LA (1999). "Mechanisms of insulin resistance in rat models of hypertension and their relationships with salt sensitivity." Journal of Hypertension **17**(9): 1229-1237.

Sena, CM, P Matafome, J Crisóstomo, L Rodrigues, R Fernandes, P Pereira and RM Seiça (2012). "Methylglyoxal promotes oxidative stress and endothelial dysfunction." Pharmacological Research **65**(5): 497-506.

Sena, CM, AM Pereira and R Seiça (2013). "Endothelial dysfunction — A major mediator of diabetic vascular disease." Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease **1832**(12): 2216-2231.

Shaker, OG and NAH Sadik (2013). "Transforming growth factor beta 1 and monocyte chemoattractant protein-1 as prognostic markers of diabetic nephropathy." Human & Experimental Toxicology **32**(10): 1089-1096.

Sharma, S, Y Sirin and K Susztak (2011). "The story of Notch and chronic kidney disease." Current opinion in nephrology and hypertension **20**(1): 56-61.

Shaw, JE, PZ Zimmet and KGMM Alberti (2006). "Point: Impaired Fasting Glucose: The Case for the New American Diabetes Association Criterion." Diabetes care **29**(5): 1170-1172.

Sheen, YJ and WH Sheu (2014). "Risks of rapid decline renal function in patients with type 2 diabetes." World Journal of Diabetes **5**(6): 835-846.

Shen, N, H Lin, T Wu, D Wang, W Wang, H Xie, J Zhang and Z Feng (2013). "Inhibition of TGF-beta1-receptor posttranslational core fucosylation attenuates rat renal interstitial fibrosis." Kidney International **84**(1): 64-77.

Shi, Y, F Cosentino, GG Camici, A Akhmedov, PM Vanhoutte, FC Tanner and TF Lüscher (2011). "Oxidized Low-Density Lipoprotein Activates p66Shc via Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1, Protein Kinase C-β, and c-Jun N-Terminal Kinase Kinase in Human Endothelial Cells." Arteriosclerosis, thrombosis, and vascular biology **31**(9): 2090-2097.

Shields, TM and CH Hennekens (2004). "Management of metabolic syndrome: aspirin." Endocrinology and Metabolism Clinics of North America **33**(3): 577-593.

Shirasaka, T, M Takasaki and H Kannan (2003). "Cardiovascular effects of leptin and orexins." American Journal of Physiology:l Regulatory, Integrative and Comparative Physiology**284**(3): R639-651.

Shirley, DG and SJ Walter (1991). "Acute and chronic changes in renal function following unilateral nephrectomy." Kidney International **40**(1): 62-68.

Siragy, HM and RM Carey (2010). "Role of the intrarenal renin-angiotensin-aldosterone system in chronic kidney disease." American journal of nephrology **31**(6): 541-550.

Slee, A (2012). "Exploring metabolic dysfunction in chronic kidney disease." Nutrition & Metabolism **9**(1): 36.

Spat, A and L Hunyady (2004). "Control of aldosterone secretion: a model for convergence in cellular signaling pathways." Physiological Reviews **84**(2): 489-539.

Stanton, A (2003). "Therapeutic potential of renin inhibitors in the management of cardiovascular disorders." American Journal of Cardiovascular Drugs **3**(6): 389-394.

Stanton, A, C Jensen, J Nussberger and E O'Brien (2003). "Blood pressure lowering in essential hypertension with an oral renin inhibitor, aliskiren." Hypertension **42**(6): 1137-1143.

Steffes, MW, DM Brown and S Michael Mauer (1978). "Diabetic Glomerulopathy Following Unilateral Nephrectomy in the Rat." Diabetes **27**(1): 35-41.

Stevens, PE and A Levin (2013). "Evaluation and Management of Chronic Kidney Disease: Synopsis of the Kidney Disease: Improving Global Outcomes 2012 Clinical Practice Guideline." Annals of Internal Medicine **158**(11): 825-830.

Stonehouse, AH, T Darsow, DG Maggs (2012). "Incretin-based therapies." Journal of Diabetes **4**(1): 55-67.

Sun, Y (2010). "Intracardiac renin–angiotensin system and myocardial repair/remodeling following infarction." Journal of molecular and cellular cardiology **48**(3): 483-489.

Sviglerova, J, J Kuncova, L Nalos, Z Tonar, D Rajdl and M Stengl (2010). "Cardiovascular parameters in rat model of chronic renal failure induced by subtotal nephrectomy." Physiological Research **59**(1): S81-88.

Sweitzer, NK (2003). "What Is an Angiotensin Converting Enzyme Inhibitor?" Circulation **108**(3): e16-e18.

Tabaei, BP, S Chamany, CR Driver, B Kerker and L Silver (2012). "Incidence of self-reported diabetes in New York City, 2002, 2004, and 2008." Preventing Chronic Disease **9**: 14.

Tan, CE, S Ma, D Wai, SK Chew and ES Tai (2004). "Can we apply the National Cholesterol Education Program Adult Treatment Panel definition of the metabolic syndrome to Asians?" Diabetes care **27**(5): 1182-1186.

Targosz-Korecka, M, GD Brzezinka, KE Malek, E Stepien and M Szymonski (2013). "Stiffness memory of EA.hy926 endothelial cells in response to chronic hyperglycemia." Cardiovascular Diabetology **12**(96): 1475-2840.

Terra, SG (2003). "Angiotensin Receptor Blockers." Circulation **107**(24): e215-e216.

Tervaert, TWC, AL Mooyaart, K Amann, AH Cohen, HT Cook, CB Drachenberg, F Ferrario, AB Fogo, M Haas, E de Heer, K Joh, LH Noël, J Radhakrishnan, SV Seshan, IM Bajema, JA Bruijn and obotRP Society (2010). "Pathologic Classification of Diabetic Nephropathy." Journal of the American Society of Nephrology **21**(4): 556-563.

Tesch, GH and AK Lim (2011). "Recent insights into diabetic renal injury from the db/db mouse model of type 2 diabetic nephropathy." American journal of physiology. Renal physiology **300**(2): 8.

Thompson, PD, D Buchner, IL Piña, GJ Balady, MA Williams, BH Marcus, K Berra, SN Blair, F Costa, B Franklin, GF Fletcher, NF Gordon, RR Pate, BL Rodriguez, AK Yancey and NK Wenger (2003). "Exercise and Physical Activity in the Prevention and Treatment of Atherosclerotic Cardiovascular Disease: A Statement From the Council on Clinical Cardiology (Subcommittee on Exercise, Rehabilitation, and Prevention) and the Council on Nutrition, Physical Activity, and Metabolism (Subcommittee on Physical Activity)." Circulation **107**(24): 3109-3116.

Tofovic, SP and EK Jackson (2003). Rat Models of the Metabolic Syndrome. Renal Disease -Methods in Molecular Medicine-. **86:** 29-46.

Tofovic SP, KH, Kost CK Jr, Bastacky S (2000). "Renal function and structure in diabetic, hypertensive, obese ZDFxSHHF-hybrid rats." Renal failure **22**(4): 387-406.

Tomilov, AA, V Bicocca, RA Schoenfeld, M Giorgio, E Migliaccio, JJ Ramsey, K Hagopian, PG Pelicci and GA Cortopassi (2010). "Decreased Superoxide Production in Macrophages of Long-lived p66Shc Knock-out Mice." Journal of Biological Chemistry **285**(2): 1153-1165.

Tortosa, A, M Bes-Rastrollo, A Sanchez-Villegas, FJ Basterra-Gortari, JM Nũnez-Cordoba and MA Martinez-Gonzalez (2007). "Mediterranean diet inversely associated with the incidence of metabolic syndrome: The SUN prospective cohort." Diabetes care **30**(11): 2957-2959.

Tremblay, ML and V Giguere (2008). "Phosphatases at the heart of FoxO metabolic control." Cell Metabolism **7**(2): 101-103.

Tuck, ML, J Sowers, L Dornfeld, G Kledzik and M Maxwell (1981). "The effect of weight reduction on blood pressure, plasma renin activity, and plasma aldosterone levels in obese patients." New England Journal of Medicine **304**(16): 930-933.

Underwood, PC and GK Adler (2013). "The renin angiotensin aldosterone system and insulin resistance in humans." Current Hypertension Reports **15**(1): 59-70.

Vague, J (1947). "La differentiation sexuelle. Facteur determinant des formes de l' obésite." Presse Médicale **55**: 339–341.

Vague, J (1956). "The degree of masculine differentiation of obesities. A factor determining predisposition to diabetes, atherosclerosis, gout and uric calculous disease." The American Journal of Clinical Nutrition **4**: 20-34.

Vaidyanathan, S, V Jarugula, HA Dieterich, D Howard and WP Dole (2008). "Clinical pharmacokinetics and pharmacodynamics of aliskiren." Clinical Pharmacokinetics **47**(8): 515-531.

Vallance, P (2001). "Importance of asymmetrical dimethylarginine in cardiovascular risk." The Lancet **358**(9299): 2096-2097.

Varin, R, P Mulder, F Tamion, V Richard, JP Henry, F Lallemand, G Lerebours and C Thuillez (2000). "Improvement of endothelial function by chronic angiotensin-converting enzyme inhibition in heart failure : role of nitric oxide, prostanoids, oxidant stress, and bradykinin." Circulation **102**(3): 351-356.

Velloso, LA, F Folli, XJ Sun, MF White, MJ Saad and CR Kahn (1996). "Cross-talk between the insulin and angiotensin signaling systems." Proceedings of the National Academy of Sciences **93**(22): 12490-12495.

Vianna, HR, CM Soares, KD Silveira, GS Elmiro, PM Mendes, M de Sousa Tavares, MM Teixeira, DM Miranda and ESAC Simoes (2013). "Cytokines in chronic kidney disease: potential link of MCP-1 and dyslipidemia in glomerular diseases." Pediatric Nephrology **28**(3): 463-469.

Viollet, B, B Guigas, N Sanz Garcia, J Leclerc, M Foretz and F Andreelli (2012). "Cellular and molecular mechanisms of metformin: an overview." Clinical Science **122**(6): 253-270.

Wang, B, JC Jha, S Hagiwara, AD McClelland, K Jandeleit-Dahm, MC Thomas, ME Cooper and P Kantharidis (2014). "Transforming growth factor-beta1-mediated renal fibrosis is dependent on the regulation of transforming growth factor receptor 1 expression by let-7b." Kidney International **85**(2): 352-361.

Wautier, JL and AM Schmidt (2004). "Protein glycation: a firm link to endothelial cell dysfunction." Circulation Research **95**(3): 233-238.

Wei, M, SP Gaskill, SM Haffner and MP Stern (1998). "Effects of Diabetes and Level of Glycemia on All-Cause and Cardiovascular Mortality: The San Antonio Heart Study." Diabetes care **21**(7): 1167-1172.

Wei, Y, JR Sowers, SE Clark, W Li, CM Ferrario and CS Stump (2008). "Angiotensin II-induced skeletal muscle insulin resistance mediated by NF-kappaB activation via NADPH oxidase." American Journal of Physiology: Endocrinology and Metabolism **294**(2): 11.

Wei, Y, JR Sowers, R Nistala, H Gong, GM Uptergrove, SE Clark, EM Morris, N Szary, C Manrique and CS Stump (2006). "Angiotensin II-induced NADPH oxidase activation impairs insulin signaling in skeletal muscle cells." The Journal of Biological Chemistry **281**(46): 35137-35146.

Whelton, PK, J He, JA Cutler, FL Brancati, LJ Appel, D Follmann and MJ Klag (1997). "Effects of oral potassium on blood pressure: Meta-analysis of randomized controlled clinical trials." Journal of the American Medical Association **277**(20): 1624-1632.

WHO (2014). "Obesity and overweight " Fact Sheet **311**.

Wild, S, G Roglic, A Green, R Sicree and H King (2004). "Global Prevalence of Diabetes: Estimates for the year 2000 and projections for 2030." Diabetes care **27**(5): 1047-1053.

Willson, TM, MH Lambert and SA Kliewer (2001). "Peroxisome proliferator-activated receptor gamma and metabolic disease." Annual Review of Biochemistry **70**: 341-367.

Wilson, PW and SM Grundy (2003). "The metabolic syndrome: practical guide to origins and treatment: Part I." Circulation **108**(12): 1422-1424.

Witteles, RM and MB Fowler (2008). "Insulin-resistant cardiomyopathy clinical evidence, mechanisms, and treatment options." Journal of the American College of Cardiology**51**(2): 93-102.

Wolk, R, AS Shamsuzzaman and VK Somers (2003). "Obesity, sleep apnea, and hypertension." Hypertension **42**(6): 1067-1074.

Wong, ND (2005). "Intensified screening and treatment of the metabolic syndrome for cardiovascular risk reduction." Preventive Cardiology **8**(1): 47-52.

Woods, LL (1993). "Mechanisms of renal hemodynamic regulation in response to protein feeding." Kidney International **44**(4): 659-675.

Wu, QQ, Y Wang, M Senitko, C Meyer, WC Wigley, DA Ferguson, E Grossman, J Chen, XJ Zhou, J Hartono, P Winterberg, B Chen, A Agarwal and CY Lu (2011). "Bardoxolone methyl (BARD) ameliorates ischemic AKI and increases expression of protective genes Nrf2, PPARgamma, and HO-1." American journal of physiology. Renal physiology **300**(5): F1180-1192.

Xydakis, AM, CC Case, PH Jones, RC Hoogeveen, MY Liu, EO Smith, KW Nelson and CM Ballantyne (2004). "Adiponectin, inflammation, and the expression of the metabolic syndrome in obese individuals: the impact of rapid weight loss through caloric restriction." The Journal of Clinical Endocrinology and Metabolism **89**(6): 2697-2703.

Yan, SF, R Ramasamy and AM Schmidt (2010). "The RAGE Axis: A Fundamental Mechanism Signaling Danger to the Vulnerable Vasculature." Circulation research **106**(5): 842-853.

Yanai, H, Y Tomono, K Ito, N Furutani, H Yoshida and N Tada (2008). "The underlying mechanisms for development of hypertension in the metabolic syndrome." Nutrition Journal **7**(10): 1475-2891.

Yki-Järvinen, H (2004). "Thiazolidinediones." New England Journal of Medicine **351**(11): 1106-1118.

Yki-Jarvinen, H and J Westerbacka (2007). "Insulin resistance, arterial stiffness and wave reflection." Advances in Cardiology **44**: 252-260.

You, H, T Gao, TK Cooper, W Brian Reeves and AS Awad (2013). "Macrophages directly mediate diabetic renal injury." American Journal of Physiology - Renal Physiology **305**(12): F1719-F1727.

Yu, HCM, LM Burrell, MJ Black, LL Wu, RJ Dilley, ME Cooper and CI Johnston (1998). "Salt Induces Myocardial and Renal Fibrosis in Normotensive and Hypertensive Rats." Circulation **98**(23): 2621-2628.

Yuan, L, X Li, GL Xu and CJ Qi (2010). "Effects of renin-angiotensin system blockade on islet function in diabetic rats." Journal of Endocrinological Investigation **33**(1): 13-19.

Zannad, F, JJV McMurray, H Krum, DJ van Veldhuisen, K Swedberg, H Shi, J Vincent, SJ Pocock and B Pitt (2011). "Eplerenone in Patients with Systolic Heart Failure and Mild Symptoms." New England Journal of Medicine **364**(1): 11-21.

Zhang, J, NA Noble, WA Border, RT Owens and Y Huang (2008). "Receptor-dependent prorenin activation and induction of PAI-1 expression in vascular smooth muscle cells." American Journal of Physiology: Endocrinology and Metabolism **295**(4): 29.

Zhou, BF, YF Wu, Y Li and LF Zhang (2005). "[The cut-off point of waist circumference for identifying metabolic syndrome in Chinese adults]." Zhonghua Xin Xue Guan Bing Za Zhi **33**(1): 81-85.

Zoja, C, A Benigni and G Remuzzi (2013). "The Nrf2 pathway in the progression of renal disease." Nephrology Dialysis Transplantation **29**(suppl 1): i19-i24.